REPUBLIC OF TURKEY
HACETTEPE UNIVERSITY
INSTITUTE OF HEALTH SCIENCES

EVALUATION OF CYTOTOXIC AND GENOTOXIC PROPERTIES OF SINAPIC ACID

Hasan HAMEED

Pharmaceutical Toxicology Programme
THESIS OF MASTER OF SCIENCES

Ankara
2014

REPUBLIC OF TURKEY

HACETTEPE UNIVERSITY

INSTITUTE OF HEALTH SCIENCES

EVALUATION OF CYTOTOXIC AND GENOTOXIC PROPERTIES OF SINAPIC ACID

Hasan HAMEED

Pharmaceutical Toxicology Programme

THESIS OF MASTER OF SCIENCES

SUPERVISOR

Prof. Dr. A. Nurşen BAŞARAN

Co-SUPERVISOR

Assoc. Prof. Sevtap AYDIN

Ankara

2014

Department : **Pharmaceutical Toxicology**

Programme : **Pharmaceutical Toxicology**

Thesis Title : **Evaluation of Cytotoxic and Genotoxic Properties of Sinapic Acid**

Stutent Name : **Hasan HAMEED**

Date of Exam : **03.09.2014**

This is to certify that this study is fully adequate in scope and quality, as a thesis for the degree of Master of Sciences in the Programme of Pharmaceutical Toxicology.

Head of Jury: **Prof. Dr. Belma GÜMÜŞEL**
(Hacettepe University)

Supervisor: **Prof. Dr. A. Nurşen BAŞARAN**
(Hacettepe University)

Member: **Prof. Dr. Ülkü ÜNDEĞER BUCURGAT**
(Hacettepe University)

Member: **Prof. Dr. Ahmet AYDIN**
(Yeditepe University)

Member: **Assoc. Prof. Ü. Pınar ERKEKOĞLU**
(Hacettepe University)

APPROVAL:

In the opinion of the above Examining Committee Members appointed by the Executive Council of the Institute of Health Sciences, this thesis was found to satisfy all the requirements as a thesis for the degree of Master of Sciences and therefore is accepted at the meeting of the Institute Executive Council of Health Sciences.

Prof. Dr. Ersin FADILLIOĞLU
Director of the Institute

ACKNOWLEDGEMENT

I greatly acknowledge the following people for their contribution in this thesis:

- I would like to express my sincere gratitude to my Supervisor, Prof. Dr. A. Nurşen BAŞARAN for her guidance, help and support of my Master study and research. This study would not have been possible without the participation of her.

- I would like to express my sincere and deep gratitude to my Co-supervisor, Assoc. Prof. Sevtap AYDIN for the all time unconditional help and support, for her patience and motivation. This study would not have been possible without the participation of her.

- I would like to express my sincere thanks to the Department of Pharmaceutical Toxicology, Faculty of Pharmacy at Hacettepe University especially those members of my thesis committee for their input, valuable discussions and accessibility.

- I would also deeply thank to Unzile YAMAN for her assistance and great friendship during past two years.

- And I would like to thank my family for their unconditional support, both financially and emotionally throughout my degree.

ABSTRACT

Hameed, H. Evaluation of cytotoxic and genotoxic properties of sinapic acid. Hacettepe University Institute of Health Sciences, M.Sc. Thesis in Pharmaceutical Toxicology. Ankara, 2014. Phenolic compounds, one of the most widely occurring groups of phytochemicals, play an important role in the growth and reproduction of plants, providing protection against pathogens and predators, also contributing towards the colour and sensory characteristics of fruits and vegetables. In humans, it is also suggested to have antioxidant, anti-inflammatory, antimicrobial, anticancer, and cardioprotective effects. Sinapic acid is a small naturally occurring hydroxycinnamic acid derivative. It is a phenolic compound and a member of the phenylpropanoid family which are assumed as therapeutically beneficial and generally non-toxic. Sinapic acid can be found in wine, vinegar, cereals, roasted coffee, green vegetables, and many other fruits. Since the data about the cytotoxicity and genotoxicity of sinapic acid is limited, in this study, the cytotoxic properties of sinapic acid were examined in different cell lines such as V79 and HeLa by neutral red uptake and 3-(4,5-dimethylthiazol-2-yl)2,5-diphenyl tetrazolium bromide (MTT) assays and also the genotoxic/antigenotoxic activities of sinapic acid were evaluated by using Comet assay (single cell gel electrophoresis) and micronucleus test in the lymphocytes and V79 cells. According to the results of this study, sinapic acid exerted different cytotoxic effects in the different cell types and at the concentrations below IC_{50} values, sinapic acid alone did not induce genotoxic damage. Sinapic acid also reduced the H_2O_2- and MMC-induced DNA damage at the non-cytotoxic concentrations.

Key Words: Sinapic acid, genotoxicity, cytotoxicity, phenolic compounds, neutral red uptake, MTT, single cell gel electrophoresis (Comet), micronucleus.

ÖZET

Hameed, H. Sinapik asitin sitotoksik ve genotoksik özelliklerinin değerlendirilmesi. Hacettepe Üniversitesi Sağlık Bilimleri Enstitüsü, Farmasötik Toksikoloji Programı Yüksek Lisans Tezi, Ankara, 2014. En yaygın görülen fitokimyasal madde gruplarından biri olan fenolik bileşikler, meyve ve sebzeleri asalak ve patojenlere karşı korumanın yanı sıra, renk ve duyusal özelliklerine de katkıda bulunarak, bitkilerin üreme ve büyümesinde önemli bir rol oynarlar. İnsanlarda da fenolik bileşiklerin antioksidan, antiinflamatuvar, antimikrobiyal, kalp koruyucu ve antikanser etkileri olduğu iddia edilmektedir. Sinapik asit doğal olarak oluşan ufak bir hidroksisinamik asit türevidir. Sağlığa faydalı ve genel olarak toksik olmadığı varsayılan fenilpropanoid ailesinden bir fenolik bileşiktir. Tahıl, kavrulmuş kahve, sirke, şarap, yeşil sebze ve diğer pek çok meyvede bulunabilir. Sinapik asidin sitotoksisitesi ve genotoksisitesi hakkında yeterli veri bulunmadığından bu çalışmada, sinapik asidin sitotoksik özellikleri V79 ve HeLa gibi farklı hücre tiplerinde Nötral Kırmızı Alımı ve 3-(4,5-dimetiltiyazol-2-il)-2,5-difeniltetrazolyum bromür (MTT) yöntemleri ile ve genotoksik/antigenotoksik etkinliği V79 hücrelerinde ve lenfositlerde Komet (Tek hücre jel elektroforezi) ve Mikroçekirdek yöntemleri ile değerlendirilmiştir. Bu çalışmanın sonuçlarına göre sinapik asit farklı hücre tiplerinde farklı sitotoksik etkiler göstermiş ve IC_{50} değerlerinin altındaki konsantrasyonlarda tek başına genotoksik hasar oluşturmamıştır. Ayrıca sitotoksik olmayan konsantrasyonlarda H_2O_2 ve mitomisin C'nin oluşturduğu DNA hasarını azaltmıştır.

Anahtar Kelimeler: Sinapik asit, genotoksisite, sitotoksisite, fenolik bileşikler, nötral kırmızı alım, MTT, tek hücre jel elektroforezi (Komet), mikroçekirdek

INDEX OF CONTENTS

ABBREVIATIONS

8-OHdG	8-Hydroxy-2′-deoxyguanosine
AAPH	2,2′-Azo-bis(2-amidinopropane) dihydrochloride
ACh	Acetylcholine
AChE	Acetylcholinesterase
ATP	Adenosine triphosphate
Aβ	Amyloid β protein
BN	Binucleated
CCl_4	Carbon tetrachloride
COX-2	Cyclooxygenase
Cu^{+2}	Copper
DNA	Deoxyribonucleic acid
$DPPH^{\cdot}$	2,2-Diphenyl-1-picrylhydrazyl
DSC	Differential scanning colorimetry
GABA	Gamma-Aminobutyric acid
H_2O_2	Hydrogenperoxide
HCAs	Hydroxycinnamic acids
HeLa	Human cervix adenocarcinoma cell line
HL-60	Promyelocytic leukemia cell line
IC_{50}	50% Inhibitor concentration
IL-1β	Interleukin-1β
iNOS	Nitric oxide synthase
KCN	Potassium cyanide
LDL	Low-density lipoprotein
LPS	Lipopolysaccharide
MN	Micronucleus
MNi	Micronuclei
MTT	3-(4, 5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NBUDs	Nuclear buds
NF-κB	Nuclear factor-kappa B

NMR	Nuclear magnetic resonance spectroscopy
NPBs	Nucleoplasm bridges
NR	Neutral red dye
NRU	Neutral red uptake assay
$O_2^{\bullet-}$	Superoxide radical
OD	Optical density
OIT	Oxidation induction temperature
$ONOO^-$	Peroxynitrite
PHA-M	Phytohaemagglutinin-M
RNA	Ribonucleic acid
ROS	Reactive oxygen species
SIN-1	3-Morpholinosydnonimine hydrochloride
STZ	Streptozotocin
T47D	Human breast cancer cell line
TNF	Tumor necrosis factor
Trolox	6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid
V79	Chinese hamster lung fibroblast cell line

FIGURES

TABLES

INTRODUCTION

Sinapic acid is a naturally occurring hydroxycinnamic acid derivative. It is a phenolic compound and a member of the phenylpropanoid family, which are assumed as therapeutically beneficial and generally not toxic.

Sinapic acid is widespread in the plant kingdom (fruits, vegetables, cereal grains, oilseed crops, some spices and medicinal plants) and in human diet. Derivatives of sinapic acid are characteristic compounds in the Brassicaceae family. Sinapic acid shows antioxidant, antimicrobial, anti-inflammatory, anticancer, and anxiolytic activity. 4-Vinylsyringol (a decarboxylation product of sinapic acid) is a potent antioxidant and antimutagenic agent, which suppresses carcinogenesis and the induction of inflammatory cytokines. Sinapine (sinapoyl choline) is considered to be an acetylcholinesterase inhibitor which might have therapeutic applications in various diseases. Mainly due to their antioxidative activities, these compounds have been suggested for potential use in food processing, cosmetics, and pharmaceutical industry.

The data about the cytotoxicity and genotoxicity of sinapic acid is limited. In this study, the cytotoxic properties of sinapic acid were examined in different cell lines such as V79 and HeLa using Neutral Red Uptake and 3-(4,5-dimethylthiazol-2-yl)2,5-diphenyl tetrazolium bromide (MTT) assays, *in vitro* cytotoxicity tests and also the genotoxic/ antigenotoxic activities of sinapic acid were evaluated in the lymphocytes and V79 cells by Comet assay (single cell gel electrophoresis) and micronucleus test.

1. THEORETICAL PRINCIPAL

1.1. General Properties of Phenolic Compounds

Phenolic compounds are a group of key plant metabolites found abundantly in fruit and vegetables. Because of their antioxidant properties, they play an important role in preventing various degenerative disorders or diseases related to oxidative damage (1). Phenolic compounds exhibit a wide range of physiological properties, such as anti-allergenic, anti-atherogenic, anti-inflammatory, anti-microbial, antioxidant, anti-thrombotic, cardioprotective and vasodilatory effects (2-4).

In nature, they are present in foods mainly as esters, glycosides and polymers, which need to undergoes enzymatic hydrolysis in the digestive tract or by the gut microflora before being absorbed. The biological properties of these phenolic compounds undergoing this degradation, are thus governed by their absorption as well as metabolism (1).

Phenolic compounds, ubiquitous in plants, are essential parts of human diet, and are of considerable interest due to their antioxidant properties. These compounds possess an aromatic ring bearing one or more hydroxyl groups and their structures may range from that of a simple phenolic molecule to that of a complex high-molecular weight polymer (5). The antioxidant activity of phenolic compounds depends on the structure, in particular the number and the positions of the hydroxyl groups and the nature of substitutions on the aromatic rings (6). Phenolic compounds are present as conjugates with mono- and polysaccharides, linked to one or more of the phenolic groups, and may also occur as functional derivatives such as esters and methyl esters (7,8).

Phenolic acids consist of two subgroups, i.e., the hydroxybenzoic and hydroxycinnamic acids. Hydroxybenzoic acids include gallic, p-hydroxybenzoic, vanillic and syringic acids, which in common have the C_6–C_1 structure. Hydroxycinnamic acids, on the other hand, are aromatic compounds with a three carbon side chain (C_6–C_3), with caffeic, ferulic, p-coumaric and sinapic acids being the most common (5).

Though phenolic compounds are present in almost all foods of plant origin,

fruits, vegetables, and beverages are the major sources of these compounds in human diet (9). Beverages such as fruit juices, tea and wines are important sources of phenolics in human diet. Most of the data available on phenolic contents are given in Table 1.1. Reductions or losses of phenolic compounds have been reported in commercial juices, and these have been attributed to commercial processing procedures (10).

A major concern expressed with regards to phenolic compounds has been in relation to the perceived role as antinutrients, particularly due to their ability to reduce digestibility of proteins, either by direct precipitation or by inhibition of enzyme activity (11). Tannins, for example form complexes with dietary proteins and carbohydrates, as well as with enzymes (12). Besides, tannins have also been shown to reduce the absorption of minerals such as iron and copper (13). However, chelation of these metals could sometimes be beneficial as this is one of the mechanisms by which phenolic compounds exert their antioxidant activity (5).

Plant phenolics are biosynthesized by two basic pathways: The shikimic acid pathway and the malonic acid pathway. The shikimic acid pathway participates in the biosynthesis of most plant phenolics. The malonic acid pathway is of more significance in fungi and bacteria than in higher plants (14).

The shikimate pathway leads to the synthesis of aromatic amino acids such as phenylalanine and tyrosine. Hydroxycinnamic acids are formed by the deamination of phenylalanine or tyrosine to yield the C_6_C_3 unit that serves as the core structure of the phenylpropanoids; the deamination is catalyzed by the enzyme phenylalanine ammonia-lyase (15,16).

In plants, tyrosine ammonia-lyase converts tyrosine into 4-hydroxycinnamic acid (also known as p-coumaric acid), which can subsequently be transformed into caffeic, ferulic, or sinapic acid. This pathway is responsible for the biosynthesis of a very large number of diverse secondary metabolites such as lignins (20).

Table 1.1. Total phenolic contents of selected vegetables, fruits, grains, and beverages

Source	**Contents (mg/100g)**			
	Caffeic Acid	Ferrulic Acid	Sinapic Acid	p-coumaric Acid
potato cooked peel	40	9.4	0.51	0.66
aubergine (eggplant)	21	0.57	ND	0.19
avocado	0.42	1.1	0.97	0.81
button mushroom	0.06	ND	ND	ND
carrot	26	1.5	ND	0.69
parsnip	1.8	2.2	0.2	0.34
radish	1.0	4.6	0.12	5.6
turnip	0.2	0.23	0.84	0.09
broccoli	1.5	4.1	8.0	0.85
Chinese cabbage	0.54	1.4	5.2	0.42
white cabbage	0.29	0.27	2.8	0.18
red cabbage	1.6	6.3	22	9.3
garlic	ND	0.63	0.66	0.09
onion	ND	0.08	0.10	0.21
soybean	0.33	12	12	12
peanut	2.4	8.7	14	103
spinach, frozen	ND	7.4	ND	3.1
sweet pepper, green	1.3	0.37	0.18	2.2
sweet pepper, red	1.2	0.55	0.38	2.4
tomato	2	0.29	ND	1.0
peach	4.9	0.11	ND	0.52
apple	0.17	0.05	0.63	0.20
banana	0.20	5.4	ND	0.46
grape	0.04	ND	0.29	1.63
cherry	17.1	0.46	ND	5.1
grapefruit	5.5	11.6	0.99	1.35
kiwi	1.5	0.19	ND	0.25
strawberry	13.09	12.17	ND	110.76
raspberry	6.39	12.103	4.28	22.46
gooseberry	35.45	6.72	ND	50.48
wild blueberry	147	4.13	ND	3.93
buckwheat grits	8.5	1.2	2.1	1.5
corn flour	2.6	38	5.7	3.1
rye bran	7.7	280	48	14
oat bran	0.54	33	9	1.2
wheat bran	3.8	300	20	9

Table 1.1. (continued) Total phenolic contents of selected vegetables, fruits, grains, and beverages

Source	**Contents (mg/100g)**			
	Caffeic Acid	Ferrulic Acid	Sinapic Acid	p-coumaric Acid
apple cider	1.16	0.076	ND	0.39
apple juice	3.6	0.10	ND	1.20
beer	0.12	0.95	0.23	0.11
black currant juice	2.6	0.78	0.78	3.2
black tea	1.42	0.16	ND	2.0
coffee	87	9.1	ND	1.27
green tea	1.34	ND	ND	1.0
orange juice	0.25	4.7	0.48	1.0
red wine	3.2	ND	ND	5.0
pasta	ND	12	1.7	0.36

(ND= Not detected) (17-19).

Hydroxycinnamic acids (HCAs) are one of the major classes of phenolic compounds found in nature (21,22). They have been shown to have beneficial effects in various human diseases, particularly atherosclerosis and cancer (23,24). They are secondary metabolites derived from phenylalanine and tyrosine, and they all have a C_6_C_3 carbon skeleton with a double bond in the side chain that may have a cis or a trans configuration. Among the most common and well-known HCAs are cinnamic acid, o-coumaric acid, m-coumaric acid, p-coumaric acid, caffeic acid, ferulic acid, and sinapic acid (25). HCAs are widely distributed in the plant kingdom (26), including many species that are consumed as food or made into beverages, such as fruits, vegetables, and grains (23,27,28). HCAs can occur freely or as components of plant polymers (29). During the past decade, HCAs received particular attention because they are the most abundant antioxidants in our diet (30,31).

1.2. Sinapic Acid

Sinapic acid is a small naturally occurring hydroxycinnamic acid derivative. It is a phenolic compound and a member of the phenylpropanoid family, the member of which are assumed as therapeutically beneficial and generally not toxic. Sinapic acid is

widespread in the plant kingdom (fruits, vegetables, cereal grains, oilseed crops, and some spices and medicinal plants) and is common in human diet. Derivatives of sinapic acid are characteristic compounds of the *Brassicaceae* family. Sinapic acid shows antioxidant, antimicrobial, anti-inflammatory, anticancer, and anxiolytic activity. 4-vinylsyringol (a decarboxylation product of sinapic acid) is a potent antioxidative and antimutagenic agent, which suppresses carcinogenesis and the induction of inflammatory cytokines. Sinapine (sinapoyl choline) is considered to be an acetylcholinesterase inhibitor which might have therapeutic applications in various disease treatments. Mainly due to their antioxidant activity, these compounds have been suggested for potential use in food processing, cosmetics, and the pharmaceutical industry (32).

However, reports on the properties of sinapic acid and its derivatives, and the suggestion by some researchers showed that these compounds may be considered for potential use as preservatives in foods, cosmetics, and in the pharmaceutical industry (33,34).

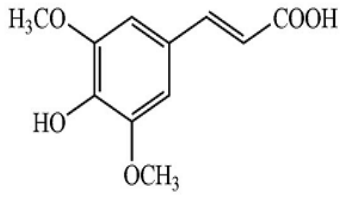

Figure 1.1. Structure of sinapic acid

1.2.1. Chemical and Physical Properties of Sinapic Acid

Sinapic acid ($C_{11}H_{12}O_5$) (3,5-dimethoxy-4-hydroxycinnamic acid) may be found in free form, but it is also found in the form of esters. Hydroxycinnamic esters are found as sugar esters (glycosides), or as esters of a variety of organic compounds (32). Sinapic acid can also form dimers with itself and ferulic acid in cereal cell walls (35-37).

It is yellow-brown crystalline powder with the molecular weight of 224.21 g/mol. The melting point of sinapic acid is 203-205 °C and it is considered incompatible

with strong oxidizing agents, strong bases. It does not dissolve well in water (38).

The most common glycoside of sinapic acid is sinapoyl glucose (1-O-β-D (glucopyranosyl sinapate) which is found throughout the many Brassicaceae species (32).

Sinapic acid, under the conditions of elevated temperature and pressure used during oil extraction from oilseeds, undergoes structural changes which result in formation of 4-vinylsyringol, as well as of syringaldehyde (3,5-dimethoxy-4-hydroxybenzaldehyde) (39). Sinapic acid and its derivatives are considered to be the most important phenolic compounds in *Brassicaceae* species (40).

The synonyms of sinapic acid are given in the below list:

- Sinapinic acid
- 3,5-dimethoxy-4-hydroxy-trans-cinnamic acid
- (2E)-3-(4-hydroxy-3,5-dimethoxyphenyl)-2-propenoic acid
- 3-(4-Hydroxy-3,5-dimethoxyphenyl) acrylic acid
- trans-4-hydroxy-3,5-dimethoxy-cinnamic acid
- trans-sinapinic acid
- 4-hydroxy-3,5-dimethoxy-cinnamic acid

1.2.2. Natural Sources of Sinapic Acid

Sinapic acid is widely distributed in the plant kingdom, it has been identified in various fruits, vegetables, cereal grains, oilseed crops, some spices and medicinal plants (32). It has been shown that Citrus fruits contain different amounts of sinapic acid, of which the lemon (*Citrus limon L.*) and Murcott orange (*C. reticulate , C. sinensis*) have the highest amounts of 72.1 μg/g and 50.1 μg/g on a dry weight basis respectively (41).

Sinapic acid has been found in various berry fruits such as strawberries (*Fragaria ananassa L.*) with 450 μg/g and American cranberries (*Oxycoccus macrocarpus*) with 210 μg/g (42,43).

Sinapic acid is also present in significant amounts in cereal grains. In rye (*Secale cereale L.)* sinapic acid represents 9% to 10% of all phenolic acids and is the most

abundant after ferulic acid. Sinapic acid content may vary from 0.07 to 0.14μg/g in different rye varieties as well (44). The content of sinapic acid in rice was 1.05 μg/g dry weight of rice, while from insoluble wild rice dietary fiber an amount 518 μg/g of alkaline extractable sinapic acid was obtained (45,46). The amount of sinapic acid in flours that made from Canadian wheat was 17 to 31 μg/g (47).

Sinapic acid was also found in different spices such as dill, anise, rosemary, sage, thyme, basil , capsicum and nutmeg (21). Sinapic acid and its derivatives are especially frequent in various Brassica vegetables: tronchuda cabbage, kale, white cabbage, turnip, broccoli, radish and leaf mustard (40).

An overview of the presence of sinapic acid various source is given in Table 1.2. (17-19).

It has been demonstrated that sinapic acid esters were the major constituents of the methanolic extract of the radish sprout (*Raphanus sativus L*.) and 3 sinapoyl esters were identified: methyl sinapate, 1,2-disinapoyl-β-D-glucopyranoside, and β-D-(3,4-disinapoyl)fructofuranosyl-α-D-(6-sinapoyl)-glucopyranoside (48). Sinapic acid and sinapine are also the main phenolics in repeseed press cake (49). Sinapic acid was found to be the most abundant phenolic acid in kale seeds (*Brassica oleracea convar. acephala*) (including free, ester, glycoside, and ester-bound forms), representing 52.4% of all phenolic acids. It was also found in kale leaves but in lower amounts (50). Wu and Prior identified 11 sinapic acid-acylated anthocyanins from red cabbage (51).

Sinapine was the major phenolic compound in mustard and sinapine constituted over 90% of the 70% of methanol extract of mustard press cake, while sinapic acid and its glycosides were found only in negligible amounts (52).

Bennett et al. (53) reported the presence of sinapine and sinapoyl acylated kaempferol, quercetin, and isorhamnetin derivatives in seeds of species other than Brassica genus members such as salad rocket (*Eruca sativa*), wall rocket (*Diplotaxis erucoides*), wild rocket (*Diplotaxis tenuifolia*), and Turkish rocket (*Bunias orientalis*) in concentrations ranging from 1000 to 10000 μg/g.

Table 1.2. Presence of sinapic acid in various edible sources

Source	**Sinapic acid content (µg/g)**
Lemon (*Citrus limon (L.) Burm.f.*)	72.1
Murcott oranges – tangor (*C. reticulata x C. sinensis*)	50.1
Pomelo (*Citrus grandis*)	22.2
Tangerine (*Citrus reticulate*)	18.1
Raspberries (*Rubus idaeus L.*)	36.89
Strawberries (*Fragaria ananassa L.*)	450.30
White onion (*Allium cepa L.*)	2.6
Red onion (*Allium cepa L.*)	2.0
Garlic (*Allium sativum L.*)	0.5
Broccoli (*Brassica oleracea L. var. italica*)	25–82
Tronchuda cabbage (*Brassica oleracea L. var. costata*)	180.1
White cabbage (*Brassica oleracea var. capitata*)	1.80
Leaf rape (*Brassica napus ssp. pabularia*)	10.41
Rye (*Secale cereale L.*)	0.07–0.14
Rice – (*Zizania aquatica L.*)	1.05
Oat – *Avena sativa L.*	56.05
Borage (*Borago officinalis L.*)	1210
Dill (*Anethum graveolens L.*)	230
Anise (*Pimpinella anisum L.*)	100
Rosemary (*Rosmarinus officinalis L.*)	690
Sage (*Salvia officinalis L.*)	280
Thyme (*Thymus vulgaris L.*)	260
Basil (*Ocimum basilicum L.)*	150
Chillies (*Capsicum*)	100
Nutmeg (*Myristica fragrans*)	348
Mace (*Myristica fragrans*)	940
Bay Leaf (*Laurus nobilis*)	135

1.2.3. Pharmacokinetics and Bioavailability of Sinapic Acid

It was demonstrated that sinapic acid was taken up in humans after consumption of a nonprocessed cereal meal to the amount of 3% of the total phenolic content of the meal. The maximum level of sinapic acid in plasma after consumption of the meal was around 40 nM, the absorption occurs mostly through the small intestine (54). The concentration of sinapic acid in human plasma after consumption of cranberry juice was found to be 1.5 μg/mL (55).

Hemery et al. (56) investigated the bioaccessibility of phenolic acids (ferulic, sinapic, and p-coumaric) from bran-rich breads. Although sinapic acid was considered to be a minor constituent in wheat grain and bran compared to ferulic acid, it significantly contributed to the antioxidant capacity of the bioaccessible bread phenolic acids, accounting for 30% to 46% of the total antioxidant capacity. Only free and conjugated forms of phenolic acids in breads were found to be bioaccessible. However sinapic acid was mostly found in the conjugated form, ferulic acid was mainly in the insoluble bound form, the higher bioaccessibility of sinapic acid was associated with its solubility (56). Sinapic acid is rapidly absorbed from the small intestine when it is in free form. However, phenolic compounds are naturally esterified in plant products and this impairs their absorption (28,57). Shivashankara et al. (58) reported that free phenolic acids are 10 to 17 times more bioavailable than esterified phenolic acids in humans.

The role of esterases present in the small intestine and in the colon of humans are shown to be able to cleave the ester bonds and may release a proportion of the hydroxycinnamic acids into the lumen, where they can then be absorbed. Furthermore, certain gut bacteria, including some already recognized as probiotic or potentially health-promoting (such as some species belonging to the genera *Bifidobacterium* and *Lactobacillus*), are involved in the release of hydroxycinnamic acids from esters and conjugates in human colon (59).

The mechanism that involved in the absorption of hydroxycinnamte is not yet well known, as it remains unclear whether their uptake is passive or active, and whether it is dependent on the intact conjugate or requires release of the aglycone at the surface of, or within, the enterocytes (60).

According to the results of previous studies, an active Na^+ gradient-driven transport mechanism may exist for cinnamic acid across the intestinal epithelium (61,62). These could include, a) the well-known sodium-glucose cotransporter, responsible for the active uptake of glucose, b) a sodium-dicarboxylate cotransporter, mainly responsible for trans-membrane transport of Krebs cycle intermediate metabolites such as succinate and citrate and c) a cotransporter which appears to be specific for cinnamic acid and ferulic acid and possibly other hydroxycinnamic acids (63). Konishi et al. (64) has suggested that that transport of hydroxycinnamates across human intestinal Caco-2 cells occurs via the monocarboxylic acid transporter.

There is limited information on the metabolism of sinapic acid compared to caffeic, ferulic, and p-coumaric acids. In general, the metabolism of polyphenols may occur in the liver, intestinal mucosa, kidney, and/or by the intestinal microflora, during which they may undergo a number of enzymatic reactions including dehydroxylation, demethylation, dehydrogenation, hydrogenation, O-methylation, sulfation, glucuronization, glutathione conjugation, and/or glycination (65).

In an *in vitro* human study, the metabolism of the major dietary hydroxycinnamates, including sinapic acid and its methyl ester was examined by Caco-2 model cells of the human small intestinal epithelium. Enterocyte-like differentiated Caco-2 cells contain esterases, able to deesterify hydroxycinnamate esters (phaseI), and intracellular sulfotransferases and uridine diphosphate glucuronosyltransferases, able to form the sulfate and the glucuronide conjugates (phase II). This study was performed by free sinapic acid and its methyl ester. In the case of methyl sinapate, the metabolites detected were sinapic acid (a confirmation of esterase activity), methyl sinapate-sulfate, and methyl sinapate-glucuronide (confirmation that methyl sinapate is a substrate for sulfotransferases and UDP glucuronosyltransferases), and in the case of sinapic acid only the sulfate conjugate was detected. Sinapic acid was also recognized as a urinary metabolite after β-glucuronidase treatment, which should be another confirmation of glucuronidation of sinapic acid through metabolism, Therefore, it can be concluded that the human small intestinal epithelium plays a role in the metabolism and the bioavailability of sinapic acid, both free and esterified, and that both forms are

susceptible to phase I and phase II of the metabolic reactions (54).

Jin et al. (66) examined the interactions of bovine serum albumin with 6 hydroxycinnamic acids, including sinapic acid, by nuclear magnetic resonance (NMR) spectroscopy techniques in combination with fluorescence and molecular modeling methods and they demonstrated that hydroxycinnamic acids were bound to bovine serum albumin mainly by hydrophobic interaction and hydrogen-bonding and also it was suggested that sinapic acid had the weakest affinity to bovine serum albumin in comparison to the other investigated compounds (chlorogenican, caffeic, m-coumaric, p-coumaric, and ferulic acids), due to the steric hindrance of methoxy substituents on the phenyl ring. It has been shown that that bovine serum albumin forms complexes with sinapic acid at pH 6.4 by electrostatic forces rather than by hydrophobic interactions (67).

1.2.4. Pharmacological Effects of Sinapic Acid

Sinapic acid shows pharmacological effects almost in all systems. Several in vitro and in vivo studies have been conducted to determine the pharmacological properties of sinapic acid and to elucidate mechanism of action of this agent. The majority range of pharmacological activities of sinapic acid has been studied and includes antioxidant, antimicrobial, anti-inflammatory, analgesic and anticancer.

- **Antioxidant Activity of Sinapic Acid**

Oxidative stress is involved in aging and in the pathology of many diseases including cancer, atherosclerosis, diabetes, and neurodegenerative, liver and kidney disorders. Dietary antioxidants may provide protection against oxidative stress-related diseases. Antioxidants are defined as organic molecules that can protect the body's cells from damage caused by free radicals and reactive oxygen species (ROS) (30).

HCAs such as sinapic acid have been described as chain-breaking antioxidants that probably act as radical scavengers (68). This function is related to their hydrogen atom donating ability and their ability to stabilize the resulting phenoxyl radicals via the conjugated system comprising the arene and the alkenyl carboxylate side chain (69-71).

Sinapic acid has been proposed as a potent antioxidant, its activity being described as higher than that of ferulic acid (3-methoxy-4-hydroxycinnamic acid) and sometimes comparable to that of caffeic acid (3,4-dihydroxycinnamic acid) (72,73). The relevance of sinapic acid in cell protection and in oxidative related diseases was already reported owing to its peroxynitrite (ONOO-) scavenging activity (74,75).

It has been shown that sinapic acid at a concentration of 20 μM was able to inhibit 33.2% of the 2,2-diphenyl-1-picrylhydrazyl ($DPPH^{\cdot}$) radical, which was comparable to the scavenging activity of caffeic acid (49.6%) and α-tocopherol (41.8%), and close to the activity of butylated hydroxytoluene (BHT) (29.2%) (76).

Nenadis et. al demonstrated that molar ratio of sinapic acid to ($DPPH^{\cdot}$) of 0.5, sinapic acid showed an inhibition of 88.4% which was close to the activities of dihydrocaffeic (94.6%), rosmarinic (93.4%), and caffeic acids (92.7%) (77).

In a study by XiaoLing, the antioxidant activity of an 8–8_-bis-lactone-dimer of sinapic acid was measured and found no $DPPH^{\cdot}$ scavenging activity at concentrations lower than 200 μM (78).

Sinapoyl glucose is regarded as the most active antioxidant component of rapeseed but later investigations on the antioxidant activity of sinapoyl glycosides gave some contrary results (79). It was found that the antioxidant activity of sinapic acid was more than sinapoyl glucose and sinapine in $DPPH^{\cdot}$ radical scavenging activity assay (52). 6-O-sinapoyl sucrose (IC_{50} = 500 μM) was also found to be less potent than sinapic acid (IC_{50} = 56 μM) as a $DPPH^{\cdot}$ radical scavenger (80).

In a study by Vuorela, the antioxidant activity of rapeseed meal and oil extracts, pure sinapic acid and 4-vinylsyringol were assessed by the 2,2-diphenyl-1-picrylhydrazyl ($DPPH^{\cdot}$) scavenging test and the result showed that the scavenging activity of sinapic acid (with 47.6% and 90.8% inhibition at concentrations of 0.5 and 1 mg/mL) respectively, was higher than that of 4-vinylsyringol (37.1% and 78.7% inhibition, respectively) (81).

On the other hand, Kylli et al. (82) evaluated the antioxidant activity of sinapoyl glucosides to assess how conjugation affects the antioxidant activity of hydroxycinnamic acids and he found that sinapoyl glucosides (methyl 2-O-sinapoyl-α-D-glucose and

methyl 6-O-sinapoyl-α- D-glucose) was slightly higher activity than sinapic acid itself in the DPPH˙ radical scavenging activity assay.

It has been shown that The $O_2^{\cdot -}$ scavenging activity of sinapic acid was considerable (IC_{50} = 17.98 mM) compared to the well-known antioxidant 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) (IC_{50} = 7.24 mM) (75).

It was suggested that sinapic acid scavenge 35.52% of generated $O_2^{\cdot -}$ at a concentration of 50 μM, sinapic acid also showed good scavenging activity in both nonenzymatic (NADH/phenazine methosulfate) and enzymatic (xanthine/xanthine oxidase) $O_2^{\cdot -}$ generating systems, with IC_{50} values of 979.2 and 70.7 μM respectively (83).

Hydroxyl radicals are among the most reactive free radicals and are probably capable of doing more damage to biological systems than any other reactive oxygen species (ROS) (84). It is indicated that the ability of sinapic acid to scavenge ˙OH radicals (IC_{50} = 3.80 mM) is comparable to that of ascorbic acid (IC_{50} = 4.56 mM) (84).

The ONOO− scavenging ability of hydroxycinnamic acids has been examined in comparison with ascorbic acid and tocopherol as endogenous antioxidants by using 3-nitrotyrosine as a marker, the results showed that sinapic acid strongly inhibited the formation of 3-nitrotyrosine better than ascorbic acid or α-tocopherol (74).

Zou et al. (75) , demonstrated that sinapic acid could efficiently scavenge native ONOO−, as well as ONOO− derived from the peroxynitrite donor 3-morpholinosydnonimine hydrochloride (SIN-1). The results showed that sinapic acid was a more potent ONOO− scavenger (IC_{50} = 0.58 mM) than penicillamine (IC_{50} = 2.93 mM), which was used as a reference compound. Sinapic acid exhibited high ONOO− scavenging activity (IC_{50} = 1.10 mM) in the presence of 25 mM Na_2CO_3, which was used as a CO_2 donor to simulate physiological conditions with high CO_2 concentrations *in vivo*.

The ability of phenolic acids including sinapic acid (at concentrations of 50 and 1000 μM) to inhibit lipid oxidation in homogeneous (bulk) and heterogeneous (emulsion) lipid systems was assessed, sinapic acid showed higher ability to inhibit formation of hydroperoxides in both bulk oil and emulsion in a concentration-dependent

manner than α tocopherol and ferulic acid (85).

The antioxidant activity of sinapic acid and other antioxidants have been studied by measuring the formation of conjugated dienes (primary oxidation products) and hexanal (secondary oxidation product) at concentrations of 50 and 500 μmol/kg in rapeseed oil. Sinapic acid, at a concentration of 500 μmol/kg, was as equally efficient as trolox and butylated hydroxyanisole at the same concentrations and it was fare more efficient than α-tocopherol. On the other hand, at 50 μmol/kg α-tocopherol showed better activity than sinapic acid, but not as good as trolox (86).

In another study by Thiyam et al. (34), the antioxidative capacity of different forms (free or bound) of phenolic compounds from rapeseed meal with respect to their concentration in stripped rapeseed oil has been measured. Sinapic acid and the free phenolic fraction of rapeseed meal (containing over 90% of sinapic acid) caused significant inhibition of the formation of hydroperoxides and propanal at concentrations of 500 μmol/kg oil, while other additives showed clearly lower activity or even a slight pro-oxidative effect.

It has been demonstrated that 4-vinylsyringol is one of the most potent antioxidant in crude rapeseed oil, with an ability to prevent lipid oxidation greater than that of many well-known antioxidants, including tocopherols, ascorbic acid, β-carotene, rutin and quercetin (87,88). According to result of a study, in the liposome model system both sinapic acid and 4- vinylsyringol were excellent inhibitors of hexanal formation at concentrations of 10 and 25 μM respectively, as well as phenolic extracts of rapeseed oil and meal at a concentration of 8.4 μg/mL (81). According to a study, in an emulsion system 4-vinylsyringol proved to be an excellent radical-chain-breaking antioxidant as determined by the β-carotene bleaching test. Its antioxidant activity exceeded that of sinapic acid by 15%. However, in polar media it was evident that decarboxylation of sinapic acid significantly decreased its reducing capacity, as 4-vinylsyringol was more than 50% less effective than sinapic acid in the ferric-reducing power assay (89).

Because of their hydrophilic nature, HCAs cannot be properly used in oil-based foods or be effective antioxidants in biological systems. Therefore, some authors suggest lipophilization (enzymatic or chemical esterification of the carboxylic group of the

hydroxycinnamic acid with a fatty alcohol) to obtain an amphiphilic compound with improved practical antioxidative properties, which should retain its original functional properties or have enhanced bioactivity and bioavailability (90).

In order to determine the influence of esterification on the antioxidant efficiency of sinapic acid, Gaspar et al. (91) examined the antioxidant activities of methyl, ethyl, propyl, and butyl sinapates. The antioxidant activity of sinapic acid and its alkyl esters was evaluated by differential scanning calorimetry (DSC) using pure linoleic acid as a lipid model system. The DSC data confirmed that sinapic acid alkyl ester derivatives are good antioxidants regarding the inhibition of lipid oxidation, since they increased the oxidation induction temperature (OIT). Sinapic acid showed an increase in OIT of 42.2 $^{\circ}$C, while sinapoyl esters showed increases in the range 51.2 to 54.1 $^{\circ}$C. The sinapic acid esters showed a bigger increase of OIT than Trolox (48.6 $^{\circ}$C). In the case of DPPH$^{\cdot}$ sinapic acid itself (IC_{50} = 32.2 μM) had a higher radical scavenging activity than its alkyl esters (48.7 to 51.9 μM). The reducing abilities of the alkyl esters were similar or lower than sinapic acid and trolox. Since trolox has been accepted as a good reducing agent in the ferric-reducing ability of the plasma assay, therefore a reducing ability at the level of trolox should be considered a remarkably good activity (91).

The oxidation of low-density lipoprotein (LDL) has been considered to be the critical point in the development of atherosclerosis and other diseases in the human body (92). Inhibition of the oxidation of LDL induced by 2,2′-azo-bis(2-amidinopropane) dihydrochloride (AAPH) gives information on the simple scavenging ability of an antioxidant against peroxyl radicals, since AAPH produces peroxyl radicals at a constant rate through its spontaneous thermal decomposition (93). Andreasen et al. (94) demonstrated that sinapic acid significantly decelerate the rate of LDL oxidation, even at the addition level of 10 μM (64% inhibition of conjugated diene formation) and completely blocked oxidation at 20 and 40 μM. The high antioxidant activity of sinapic acid was related to its capacity to chelate Cu^{2+} (95,96).

In another study, sinapic acid showed greater antioxidant activity (28%) than 4 - vinylsyringol (7.5%) in the LDL model system at 10 μM concentration, while at higher concentrations (25 μM) 4-vinylsyringol (97.1%) was slightly more effective than sinapic

acid (95.3%) (81).

The effect of esterification of sinapic acid on their lipophilicity and their protective effect against 2 types of oxidative stress was analyzed: Cu^{2+}-catalyzed peroxidation of LDL and radical attack on erythrocyte membranes by AAPH. The effects of sinapic acid and ethyl sinapate on the susceptibility to oxidation of LDL were investigated by determination of the increase in the negative charge of the LDL apoprotein and the hydroperoxide concentration. The result showed that Ethyl sinapate was a more potent inhibitor of LDL modification than sinapic acid; at 10 μM it preserved 76% of the native apoprotein fraction, while sinapic acid preserved 59%. Esterification of sinapic acid resulted in a stronger inhibition of hydroperoxide formation when measured at a concentration of 5 μM (97).

Erythrocytes also represent good models for investigation of cell injury induced by free radicals and the protective effect of antioxidants, since free radicals such as AAPH induce hemolysis due to oxidative damage to the erythrocyte membrane (98). It was found that sinapic acid inhibited AAPH induced hemolysis by 50% at 4.5 μM compared to cinnamic acid or caffeic acid that inhibited AAPH induced hemolysis by 50% at 6.8 and 7.2 μM, respectively. Furthermore, ethyl sinapate had a similar activity to that of sinapic acid (5.0 μM) (97).

- **Antiinflammatory Effects of Sinapic Acid**

In an animal study, it has been demonstrated that sinapic acid exert anti - inflammatory effect which results from inhibition of lipopolysaccharide (LPS) induced expressions of inducible nitric oxide synthase (iNOS) and cyclooxygase (COX)-2 at the protein levels, and iNOS, COX-2, TNF-R, and IL-1β mRNA expression in RAW 264.7 in a dose dependent manner. The result suggested that repression of iNOS and COX-2 induction and of the productions of pro-inflammatory cytokines by sinapic acid may represent important mechanisms involved in the inhibition of paw edema formation in rats and mice by serotonin or carrageenan (99).

- **Anticancer Effects of Sinapic Acid**

There is some data about the anticancer effects of sinapic acid in the literature. According to a study, sinapic acid exerted an inhibitory effect against tumorigenic colon cells, but had a low influence on breast cancer cells. On the other hand, sinapic acid showed antiproliferative action (ability to prevent, or retard, the spread of malignant cells into surrounding tissue) on the human breast cancer (T47D) cell line (46). Supportively, a time-dependent and dose-dependent inhibitory effect was indicated on T47D cancer cells that were treated by sinapic acid. It decreased cell proliferation by 20%, with an IC_{50} value of 7×10^{-11} M (100).

Alternatively, Cao et al. (101) treated *Helicobacter pylori*-infected Mongolian gerbils with an extract which contained 4-vinylsyringol. Expression of interleukin-1β (IL-1β), tumor necrosis factor-α (TNF-α), COX-2 and iNOS mRNA in the gastric mucosa, and serum 8-hydroxy-2′-deoxyguanosine (8-OHdG), anti-*Helicobacter pylori* IgG and gastrin levels were significantly lower in 0.1% treated groups. Furthermore, the incidence of gastric adenocarcinomas was markedly reduced in the treated group (15.0%) compared to the control group (39.4%). These data indicates 4-vinylsyringol to be effective for suppressing gastric epithelial cell proliferation and gastric carcinogenesis in *Helicobacter pylori*-infected Mongolian gerbils.

Antimutagenic properties of 4-vinylsyringol isolated from crude rapeseed oil and pure sinapic acid was tested by the constant flux method. Sinapic acid inhibited mutation by 43% at 10 μM and by 20% at 100 μM, while 4-vinylsyringol exhibited a potent antimutagenic effect at concentrations >8 μM in a dose dependent manner. It is supposed that the antimutagenic potency of 4-vinylsyringol is a consequence of its $ONOO^-$ scavenging ability. 4-Vinylsyringol had a potential protective effect against DNA cleavage induced by $ONOO^-$. In the same investigation, it was demonstrated that 4-vinylsyringol also had effects on ONOO−-induced bactericidal action. The bactericidal activity of ONOO− was evaluated with the constant flux method, using Salmonella typhimurium culture. 4-Vinylsyringol showed protective activity against the bactericidal action of ONOO− at very low concentrations between 0.1 and 1 mM. As a result, it was suggested that 4-vinylsyringol suppresses ONOO—induced cell damage,

thus preventing apoptosis of bacterial and mammalian cells, as well as preventing plasmid DNA-strand breakage (102).

- **Anxiolytic Effects of Sinapic Acid**

It is suggested that sinapic acid is an important anxiolytic (anti-anxiety) agent. Based on electrophysiological investigations, it was found that sinapic acid might act like the agonist of a specific gamma-aminobutyric acid (GABA) receptor (ligand-gated ion channel: $GABA_A$) and that it was the most effective at a dose of 4 mg/kg. It was concluded that the anxiolytic-like effects of sinapic acid are mediated via $GABA_A$ receptors and potentiating Cl^- currents (103).

- **Antimicrobial Effects of Sinapic Acid**

The antibacterial activity of sinapic acid has been demonstrated in various studies on both plant and human pathogens. It was found that sinapic acid has inhibitory action against the growth of *Xylella fastidiosa*, a pathogenic bacterium that causes diseases in many crop species, leading to considerable economic losses (104). It has been showed that that sinapic acid displays bactericidal activity against *Salmonella enterica ssp. enterica*, the most common cause of human foodborne illness (105).

It was found that sinapic acid fraction isolated from the ethanolic extract of rapeseed to be highly effective against the growth of Gram-negative (*Escherichia coli, Enterobacter aerogens,* and *Pseudomonas fluorescens*) and Gram-positive (*Bacillus subtilis, Bacillus cereus, Streptococcus lactis,* and *Streptococcus cremoris*) bacteria (106).

The antimicrobial activity of sinapic acid to *fastidiosa* strains that use grape or almond as hosts was expressed as the minimal inhibitory concentration (MIC) and values of 2000 and 800 μM were obtained. The results showed that sinapic acid inhibited the growth of *Erwinia carotovora*, a common cause of decay in stored fruits and vegetables at a concentration of 1 mg/mL in nutrient broth (107).

The minimal inhibitory concentrations (MIC) of sinapic acid for *Bacillus subtilis, E. coli*, and *Pseudomonas syringae* were 2 mM, 4 mM, and 8 mM, respectively,

determined by the microdilution method in nutrient broth (108) . In another study, MIC values were found as 2.2, 2.0 and 1.9 mM (109).

Engels et. al approved the antibacterial activity of sinapic acid against *Bacillus subtilis* (MIC = 0.3 g/L), *E. coli* (MIC = 0.7 g/L) and *Staphylococcus aureus* (MIC = 0.3 g/L) and assessed its effects against *Listeria innocua* (MIC = 0.3 g/L), *Listeria monocytogenes* (MIC =0.2 g/L) and *Pseudomonas fluorescens* (MIC = 0.6 g/L) (110).

- **Neuroprotective Effects of Sinapic Acid**

Acetylcholinesterase (AChE) regulates the concentration of the transmitter by hydrolyzing and inactivating acetylcholine (ACh). Inhibitors of AChE have therapeutic applications in diseases associated with the deficiency of ACh such as Alzheimer's disease, senile dementia, ataxia, myasthenia gravis, and Parkinson's disease. Sinapine (sinapoyl choline) was considered and investigated as a potential AChE inhibitor as a consequence of its structural similarity to ACh (111).

He et al. (112) described that sinapine could significantly inhibit AChE activity *in vitro*, being more effective in a cerebral homogenate (IC_{50} = 3.66 μM) than in blood serum (IC_{50} = 22.1 μM) of rats. Because of the high content of sinapine in kale and tronchuda cabbage seeds, their aqueous extracts were tested. Both extracts exhibited a concentration-dependent AChE inhibitory activity. For a kale extract containing 17.5 μg/mL of sinapine, IC_{50} was 3438 μg/mL. For a tronchuda cabbage seed extract (containing 22.8 μg/mL sinapine), the IC_{50} obtained corresponded to 3399 μg/mL. However, in this study, it is demonstrated that sinapine significantly inhibited AChE in rat cerebral homogenate and in rat blood serum. It was suggested that sinapine might act as a competitive inhibitor of AChE, since it possesses quaternary nitrogen that could bind reversibly to the site on the enzyme where the quaternary ammonium of ACh binds (112,113).

Lee et al. (114) established that sinapic acid attenuated amyloid β $(Aβ)_{1–42}$ protein-induced Alzheimer's disease. The results suggested that sinapic acid could be used as an effective treatment for Alzheimer's disease. The neuroprotective activity of sinapic acid was derived from its anti-apoptotic, anti-inflammatory and radical-

scavenging properties.

It was found that sinapic acid (10mg/kg) attenuates kainic acid-induced hippocampal neuronal damage in mice. The results suggested that the potential therapeutic effects of sinapic acid in brain was due to its anti-convulsive activity through $GABA_A$ receptor activation and radical scavenging activity (115).

It has also been demonstrated that sinapic acid inhibited potassium cyanide (KCN)-induced hypoxia or carotid-artery ligation-induced mortality. In the same study, sinapic acid significantly inhibited CO_2-induced impairment which was suggested that the anti-amnesic effect of sinapic acid maybe achieved by preventing the neuronal death or damage resulting from hypoxia (116).

The neuroprotective effect of sinapic acid was investigated by using 4 vessel occlusion model induced ischemia and cognitive impairments in rats. The result indicated that sinapic acid (1, 3, and 10mg/kg) confers significant neuroprotection especially for ischemic hippocampal neurons. However, this study is the first work that investigated neuroprotection under sinapic acid treated conditions in global cerebral ischemia (117).

- **Cardioprotective Effects of Sinapic Acid**

In an *in vitro* study on rats, the protective effects of sinapic acid on lysosomal dysfunction in isoproterenol induced myocardial infarcted rats were assessed. Pre-and-co-treatment with sinapic acid (12mg/kg) normalized all the biochemical parameters and reduced myocardial infarct size in myocardial infarcted rats. The possible mechanisms for the observed effects are attributed to sinapic acid free radical scavenging and membrane stabilizing properties (118).

- **Hepatoprotective Effects of Sinapic Acid**

The hepatoprotective effects of sinapic acid in carbon tetrachloride (CCl_4) induced acute hepatic injury in rats was assessed. Sinapic acid treatment reduced CCl_4-induced abnormalities in hepatic histology, serum alanine transaminase and aspartate transaminase activities, and liver malondialdehyde levels. In addition, sinapic acid

treatment significantly attenuated the CCl_4-induced production of inflammatory mediators, including tumor necrosis factor-alpha, interleukin-1β mRNA levels and increased the expression of nuclear factor-kappa B (NF-κB p65) (119).

According to the study by Wilson et. al, the effects of sinapic acid on certain biochemical markers and histology of liver and kidney in normal and streptozotocin (STZ) -induced diabetes in Wistar rat were evaluated. Oral administration of sinapic acid for a period of 35 days restored all these biochemical parameters (blood urea, serum creatinine, uric acid, total protein and albumin albumin/globulin ratio) and histopathological changes that occurred in liver and kidney to near normal levels (120). Chronic exposure to arsenic has been linked to numerous human diseases such as diabetes, atherosclerosis, cardiovascular diseases, hyperkeratosis and various types of cancers. Since arsenic targets ubiquitous enzyme reactions, it affects nearly all organ systems in animals and humans. Sinapic acid was suggested to have protective role against arsenic induced toxicity in rats. In the study, rats were orally treated with arsenic alone (5 mg/kg/day) plus sinapic acid at different doses (10, 20 and 40 mg/kg) for 30 days. It is indicated in this study that 40mg/kg sinapic acid has higher efficacy to normalize hepatic enzymes and histopathology of liver (121).

- **Antihyperglycemic Effects of Sinapic Acid**

It was found that sinapic acid has a potential antihyperglycemic effect in streptozotocin -induced diabetic rats. Pre-and-co-treatment with sinapic acid normalized all the biochemical parameters (plasma glucose, insulin, C-peptide, levels of blood hemoglobin, glycosylated hemoglobin, the activities of carbohydrate metabolizing enzymes hexokinase, glucose-6-phosphatase, fructose-1and 6-bisphosphatase) (122).

1.2.5. Toxic Effects of Sinapic Acid

There are very few studies related to the cytotoxicity and genotoxicity of sinapic acid.

Sinapic acid has been shown to exert slightly higher cytotoxic activity than of its ester derivate in the superoxide scavenging test (80) . The treatment of sinapic acid did

not induce cytotoxic effect on human neuroblastoma cells (SH-SY5Y). This result showed that sinapic acid could be used safely (117).

The effectiveness of sinapic acid as pBR322 plasmid DNA-cleaving agents in the presence of Cu^{2+} ions was investigated. Sinapic acid was remarkably more effective at causing DNA damage than other phenolic compounds toward human promyelocytic leukemia (HL-60) cell proliferation. Addition of exogenous Cu^{2+} ions resulted in an effect dichotomy on cell viability depending on the concentration of sinapic acid, that is, low concentrations of sinapic acid enhanced the cell viability, and conversely, high concentrations of sinapic acid almost completely inhibited the cell proliferation. The good correlation between the DNA damaging activity and the oxidative potential of the sinapic acid indicates that the electron transfer between HCAs and Cu^{2+} plays a crucial role in the reaction (123,124). The cytoprotective effect of sinapic acid was examined by using both apoptosis and necrosis endpoints. Sinapic acid exhibited protection against H_2O_2-mediated cytotoxicity in a dose dependent manner (125).

Antiproliferative and apoptotic effects of selective phenolic acids on T47D human breast cancer cells were also demonstrated that phenolic acids including sinapic acid showed a dose dependent and time dependent antiproliferative effect on T47D cells. It was also reported that the inhibitory effect of sinapic acid on tumoral proliferation might be due to its direct interaction with the aryl hydrocarbon receptor, the nitric oxide synthase inhibition and its pro-apoptotic effect (100).

In an *in vitro* study by Lee-Manion et al. (126), the antigenotoxic effects of hydroxycinnamic acids such as sinapic acid were evaluated by using comet assay. The result showed that sinapic acid has antigenotoxic effect on human adenocarcinoma colon cells.

However, up to now, there is not enough data about the cytotoxicity and genotoxicity of sinapic acid; the available data are limited and contrary.

1.3. Cytotoxicity Assays

1.3.1. Neutral Red Uptake Assay

The neutral red uptake (NRU) assay is one of the most used tests in cytotoxicity tests with many biomedical and environmental applications such as toxicity of chemical agents, viral cytopathogenicity and treatment effectiveness, estimation of cytotoxic T-lymphocyte populations, phototoxicity, tumoral cell growth, chemotherapy effectiveness and determination of toxins in biological and environmental samples or biotechnological products. It is also used in ranking chemicals according to their toxic potencies. This test is based on the ability of viable cells to incorporate and bind to the neutral red dye (NR). NR (toluene red, $C_{15}H_{17}C_lN_4$) is a weakly cationic dye, which penetrates cell membranes by nonionic passive diffusion and concentrates in the lysosomes. NR dye binds to the phosphate groups or anionic groups of lysosomes by electrostatic hydrophobic bonds. Then by using an acidified ethanol solution, neutral red dye is extracted from the viable cells and the absorbance of the solubilized dye is quantified by spectrophotometric method (127).

The uptake of dye depends on the production of adenosine triphosphate (ATP). cells which are able to produce ATP have the capacity to maintain pH gradients. At physiological pH, the net charge of the dye is close to zero, and dye is enabling to penetrate the membranes of the cells. There is also a proton gradient in the lysosomes, which enable them to maintain pH lower than pH of the cytoplasm. Thus, the dye is charged and retained inside the lysosomes. But when the cells die or the pH gradient is reduced, the dye is not charged and extracted from the cells (127).

The absorbance of the solubilized dye is quantified by using spectrophotometry at 540 nm wavelengths. An increase or decrease in the number of cells results in a concomitant change in the amount of dye incorporated by the cells in the culture. This indicates the degree of cytotoxicity caused by the test material (128).

1.3.2. (3-(4,5-Dimethylthiazol-2-yl)2,5-diphenyl Tetrazolium Bromide) (MTT) Assay

The MTT (3-(4,5-dimethylthiazol-2-yl)2,5-diphenyl tetrazolium bromide) cleavage assay is a method widely used for measuring cell survival and proliferation of living cells and cytotoxicity of substances. The MTT assay described by Mosmann et. al for measuring cell survival/proliferation, has been used successfully to quantitate macrophage-mediated cytotoxicity (129).

MTT and other tetrazolium salts are reduced to form water-insoluble violet formazan crystals within the cell by the respiratory chain and other electron transport systems (130). Although in various cell lines, over 50% of the dye penetrating the cell membrane can be reduced by non-mitochondrial, cytosolic and microsomal enzymes. Despite the variability of the sites of MTT reduction within the cell, it is assumed that the MTT reduction rate is closely related to the number of actively respiring cells in the cell culture (131).

The amount of these crystals can be determined colorimetrically and serves as an estimate for the number of mitochondria and hence the number of living cells in the sample (131). These features can be taken as an advantage of in cytotoxicity or cell proliferation assays, which are widely used in immunology, toxicology, and cellular biology (132). In most reports on the MTT method the optical density (OD) measurement reaches a maximum after 4 to 8 h and remained constant thereafter, however, incubation periods up to 24 h did not have a negative influence (133). Another change to the original protocol was the removal of the medium prior to the measurement. This resulted in improvement of the accuracy and reliability (131).

Mosmann et al. (129) established that the formazan generated would depend on cellular metabolism, this result may reflect the metabolic state of the cells, being worse 2 h after the splitting procedure than after 48 h culture. These results suggest a more general conclusion that a dose-response curve evaluated at one point in time for a given cell line does not necessarily apply to any other time; It should not only depend on the cell line but also on the culture time required for a given test.

The cytotoxic activity measured by the MTT method was specific and a

reflection of the pharmacologically induced activation state of the effectors. MTT assay was used for measuring the *in vitro* cytotoxic activity of activated macrophages towards sensitive target cells. This method is sensitive and reproducible, as is the 3H-TdR uptake assay, but is easier to perform and to evaluate. In fact, it can be carried out entirely in 96-well microtiter plates, since it does not require the use of radioactive markers or the transfer of samples to counting vials. In conclusion, it is a rapid procedure and large amounts of data can be generated and processed in a short time (134).

1.4. Genotoxicity Assays

1.4.1. Single Cell Gel Electrophoresis Assay (Comet Assay)

The Single Cell Gel Electrophoresis assay (known as the Comet assay) is a simple, cheap, and extremely sensitive method for measuring deoxyribonucleic acid (DNA) strand breaks and DNA damage induced by chemicals at the individual eukaryotic cells. This molecular method measures deoxyribonucleic acid strand breaks. The term "comet" refers to the pattern of DNA migration through the electrophoresis gel, which often resembles a comet (135).

The alkaline comet assay (single cell gel electrophoresis) is a simple, economical and versatile assay for measuring DNA strand breaks at the level of individual cells. After lysis of agarose-embedded cells the resulting ‘nucleoids’, comprising supercoiled DNA, are electrophoresed and DNA containing breaks extends towards the anode. On examination by fluorescence microscopy, the extended DNA forms a comet-like tail, the relative intensity of which reflects the break frequency. Digestion of nucleoids with enzymes that convert specific lesions to DNA breaks allows measurement of oxidised pyrimidines and purines, alkylated bases, and UV-induced pyrimidine dimers. In this technique, cells are embedded in a low-melting-point agarose suspension on a microscope slide. The agarose forms a matrix of carbohydrate fibers that encapsulate the cells and anchoring them on slide. The agarose is considered to be osmotic- neutral, therefore solutions can penetrate the gel and affect the cells. Cells are lysed with a solution which consists of a highly concentrated aqueous salt and a detergent (such as

Triton X-100) to form nucleoids containing supercoiled loops of DNA linked to the nuclear matrix. Then the pH of the lysis solution can be adjusted (usually between the neutral and alkaline pH) The most common form of the assay is the alkaline comet assay (135,136).

The aqueous salt disrupts proteins and their bonding patterns within the cell as well as disrupting the ribonucleic acid (RNA) content of the cell. The detergent dissolves the cellular membranes. The cells are destroyed through the action of the lysis solution. All proteins, RNA, membranes and cytoplasmic and nucleoplasmic constituents are disrupted and diffuse into the agarose matrix. The DNA of the cell remains, and unravels to fill the cavity in the agarose that the whole cell formerly filled. This structure is called nucleoid (a general term for a structure in which DNA is concentrated) (137).

After lysis of the cells (typically 1 to 2 hours at $4^{\circ}C$), the slides are washed in distilled water to remove all salts and immersed in an electrophoresis solution. In alkaline conditions the DNA double helix is denatured and the nucleoid becomes single stranded (137).

DNA damage is detected after electrophoresis of single cells embedded in agarose where, in alkaline pH (pH>13),the broken DNA strands move towards the anode and result in comet shapes, observed by fluorescence microscopy. Indeed, the electric current pulls the charged DNA from the nucleus and broken DNA fragments migrate. The intensity of the comet tail relative to the head shows the number of DNA breaks (135,137).

In this method, undamaged DNA retains a highly organized association with matrix proteins in the nucleus. In damaged DNA, this organization is disrupted. The individual strands of DNA lose their compact structure and relax, expanding out of the cavity into the agarose. When the electric field is applied the DNA, which has an overall negative charge, is drawn towards the positively charged anode. Undamaged DNA strands are too large and do not leave the cavity, whereas the damaged fragments, move in a given period of time. Therefore, the amount of DNA that leaves the cavity is a measure of the amount of DNA damage in the cell (135).

The image analysis measures the intensity of the fluorescence for the whole nucleoid and the fluorescence of the migrated DNA and compares the two signals. The more damaged DNA presents stronger signal. The overall structure resembles a comet shape with a circular head corresponding to the undamaged DNA that remains in the cavity and a tail of damaged DNA. The brighter and longer the tail, the higher the level of damage. The Comet assay is one of the most important assays in toxicology today and the assay can be used for genotoxicity testing of novel compounds and ecotoxin, human bio monitoring and epidemiology and basic research in to DNA damage and repair (137).

1.4.2. The Cytokinesis-Block Micronucleus Assay (Micronucleus (MN) Assay)

The cytokinesis-block micronucleus assay is successfully recognized as one of the most successful and reliable assays for screening various genotoxic compounds in both *in vivo* and *in vitro* cytogenetic studies especially in chromosome damages, and also in various research fields such as nutrigenomics and pharmacogenomics (138).

A micronucleus (MN) is the third nucleus that is formed during the metaphase/ anaphase transition of mitosis or meiosis (cell division). Micronuclei (Howell-Jolly bodies) are cytoplasmic bodies, which are expressed in dividing cells. Some divided cells contain chromosome breaks lacking centromeres (acentric fragments) or whole chromosomes that are unable to travel to the spindle poles during the anaphase of mitosis. MN may arise from a whole lagging chromosome or an acentric chromosome fragment detaching from a chromosome after breakage (clastogenic event) which do not integrate in the daughter nuclei (138).

The formation of MN results in the daughter cell lacking a part or all of a chromosome. These chromosome fragments or whole chromosomes normally develop nuclear membranes and form as micronuclei. After cytokinesis, one daughter cell ends up with one nucleus and the other ends up with one large and one small nucleus, i.e., micronuclei. There is a chance of more than one micronucleus forming when more genetic damage has happened (139).

DNA damage events are scored specifically in once-divided binucleated (BN)

cells and include:

a) Micronuclei (MNi), a biomarker of chromosome breakage or whole chromosome loss.

b) Nucleoplasm bridges (NPBs), a biomarker of DNA misrepair or telomere end-fusions,

c) Nuclear buds (NBUDs), a biomarker of elimination of amplified DNA or DNA repair complexes.

Scoring of micronuclei can be performed easily on different human cell lines such as lymphocytes, fibroblasts and exfoliated epithelial cells. An *in vitro* analysis of lymphocytes in the presence of cytochalasin-B (Cyt-B), allows distinguishing easily between mononucleated cells that did not divide and binucleated cells which completed nuclear division. Cyt-B is an inhibitor of actin microfilament. These microfilaments are required for the completion of cytokinesis (138).

Indeed, in these conditions the frequencies of mononucleated cells provide an indication of the background level of chromosome/genome mutations accumulated *in vivo* and the frequencies of binucleated cells with MN a measure of the damage accumulated before cultivation plus mutations expressed during the first *in vitro* mitosis. Cytostatic effects are measured via the proportion of mono-, bi- and multinucleated cells. Further information regarding mechanisms leading to MNi, NPBs and NBUDs formation is obtained using centromere and telomere probes (139).

2. EXPERIMENTAL SECTION

The study was approved by Hacettepe Ethical Committee (Date: 08.01.2014 and Issue no: GO/1439) according to the ‘‘Declaration of Helsinki’’. All subjects participated in the study voluntarily and written consent (in Turkish) was obtained before blood samples were drawn.

2.1. Chemicals

3-(4,5-dimethylthiazol-2-yl)2,5-diphenyl tetrazolium bromide (MTT)	Sigma
Cytochalasin B (Cyt-B)	Sigma
Dimethyl Sulfoxide (DMSO)	Sigma
Dimethylformamide (DMF)	Sigma
Dulbecco’s Modified Eagle’s Medium (DMEM)	Sigma
Dulbecco’s Phosphate Buffered Saline, sterile	Merck
Ethanol	Sigma
Ethidium Bromide (EtBr)	Sigma
Ethylene Diamine Tetra Acetic Acid Disodium (Na_2EDTA)	Sigma
Fetal Calf Serum (FCS)	Biological Industries
Formaldehyde	Sigma
Giemsa	Merck
Glacial Acetic Acid	Merck
HeLa (Human Cervix Adenocarcinoma) Cell Line	Americal Type Culture Collection,

	Rockville, Md
Heparin (Sodium Salt)	Sigma
Histopaque-1077	Sigma
Hydrochloric Acid (37%)	Merck
Hydrogen Peroxide (35%)	Merck
L-Glutamine	Biological Industries
Low Melting Point Agarose (LMA)	Sigma
Methanol	Sigma
Mitomycin C (MMC)	Sigma
Neutral Red (NR) Dye	Sigma
Nitric Acid (HNO_3)	Sigma
N-Lauryl Sarcosinate Sodium Salt	Sigma
Normal Point Melting Agarose (NMA)	Sigma
Penicillin–Streptomycin	PAA The Cell Culture Company
Phosphate Buffered Saline (PBS) tablets	Sigma
Phytohaemagglutinin-M (PHA-M)	Biological Industries
Potassium Chloride (KCl)	Sigma
Potassium Dihydrogen Phosphate (KH_2PO_4)	Sigma
Potassium Hydrogen Phosphate (K_2HPO_4)	Sigma
RPMI-1640	Sigma
Sinapic Acid	Sigma
Sodium Chloride (NaCl)	Merck

Sodium Dodecyl Sulfate (SDS)	Sigma
Sodium Hydroxide (NaOH)	Merck
Thiazolyl Blue Tetrazolium Bromide	Sigma
Tris	Sigma
Triton X-100	Sigma
Trypan Blue	Sigma
Trypsin-EDTA	Biological Industries
V79 (Chinese Hamster Fibroblast) Cell Line	Americal Type Culture Collection, Rockville, Md

2.2. Materials and Apparatus

Centrifuge	Hettich Rotina 420R
Coverslip (24x60mm)	Marienfeld
Deep Freeze (-20^{o}C)	Ariston
Deep Freeze (-80^{0}C)	Revco
Deionized Water Device	Barnstead
Distilled Water Device	MES ultrapure®
Electrophorese	Biometra Analitik
Electrophorese Power Supply	Power Pack P 25
Etuve	Dedeoğlu
Fluorescent Microscope	Leica
CO_2 Incubator	Heraeus Instruments

Inverted Microscope	Leica
Laminar Flow	Heraeus
Magnetic Mixer	Stuart Scientific
Micro Centrifuge	Heraeus
Micropipettes 1-10 µl, 0, 5-40 µl, 40-200 µl, 200-1000 µl, 1-5ml	Finnpipette,Gilson, Biohit
Microfilter	Millipore® Merck
Neubauer Slide	Marienfeld
PH meter	Cyberscan
Scale	Schimadzu Libror
Slides (26x76mm)	Marienfeld
Spectrophotometer	Schimadzu Libror
Ultrasonic Bath	Transsonic 460/H
Vortex	Heidolph 2000
Water Bath	Termal® Laboratory Tools
Comet Analysis Software, Version 3.0	Kinetic Imaging Ltd, Liverpool, UK

2.3. Solutions of Sinapic acid

- 250 mM of Sinapic Acid Solution

56 mg of sinapic acid is dissolved in 1 ml of PBS containing 0.5 ml of DMSO. Before use, the chemical solution is filtered using Millipore filter.

- 100 mM of Sinapic Acid Solution:

22.4 mg of sinapic acid is dissolved in 1 ml of PBS containing 0.10 ml of DMSO. Before use, the chemical solution is filtered using Millipore filter.

- 20 mM of Sinapic Acid Solution

400 µl of 100 mM sinapic acid solution is dissolved in 1600 µl of DMEM supplemented with 10% fetal calf serum (FCS) and 2% Penicillin-streptomycin.

- 10 mM of Sinapic Acid Solution

200 µl of 100 mM sinapic acid solution is dissolved in 1800 µl of DMEM supplemented with 10% fetal calf serum (FCS) and 2% Penicillin-streptomycin.

2.4. Cytotoxicity Assays

2.4.1. Solutions of Neutral Red Uptake (NRU) Assay

- Neutral Red Stock Solution (4 mg/ml)

20 mg of NR powder is dissolved in 5 ml distilled water. The solution must be kept in darkness at 4^0C temperature. The NR Stock Solution can be stored in the dark at 4 ^{0}C for up to one month.

- Neutral Red Standard Solution (50 µg/ml)

625 µl of NR stock solution is mixed with 50 ml of DMEM. It is filtered from 0.2 µm Millipore® filters for sterilization. The NR standard solution must be prepared 18 hours before the experiment and must be kept in incubator at 37ºC ± 1ºC, 90 % ± 5 % humidity, 5.0 % ± 1 % CO_2/air.

- Neutral Red Fixation Solution

100 ml of ethanol and 2 ml of acetic acid are mixed with 98 ml distilled water.

2.4.2. Procedure of NRU Assay

The cytotoxicity of sinapic acid was performed in V79 and HeLa cells by NRU assay following the protocols described by Virgilio et al. (140) and Saquib et al. (141) .

All the procedures must be carried out in the laminar flow safety cabinet. The safety cabinet has been thoroughly cleaned and all equipments have been wiped down with 70% ethanol before use.

1. V79 and HeLa cells were seeded in the culture medium (DMEM) supplemented with 10% fetal calf serum and 1% penicillin-streptomycin solution. Cells were cultured in 75 ml cell culture flasks.
2. Cells were incubated at 37ºC ± 1ºC, 90 % ± 5 % humidity, 5.0 % ± 1 % CO_2/air for 24 hours.
3. After 24 hours, medium were aspirated with the aspirator pump. 5 ml of sterilized warm PBS at 37^0C was added to the flask to wash the cells, and PBS was aspirated. Then 5 ml of warm trypsin-EDTA (10X) at 37^0C was added to the flask to wash the cells and then the trypsin was aspirated. 5 ml of warm trypsin-EDTA (10X) at 37^0C was added to the flask for trypsinization of the cells. The cells were incubated for 5 min at 37^0C in the incubator.
4. After incubation, cells were detached from the flask and checked under the microscope to ensure all cells being detached.
5. 10 ml culture medium at 37 ^{0}C with fetal calf serum was added to the flasks to stop the reaction. The cell suspension was transferred to a sterile tube.
6. Cells were centrifuged for 5 min at 1200 rpm. The supernatant was discarded by aspiration. Cells were suspended in 2 ml of culture medium.
7. 10 µl of cells were mixed with 90 µl of trypan blue and the cells were counted using Neubauer slide.
8. 10000 cells/well in 200 µL culture medium were seeded in a 96-well plate. Each plate was controlled under a phase contrast microscope.
9. Plates were incubated for 24 hours in a humidified atmosphere of 5% CO_2 in air at 37^0C.
10. After incubation, the viability and also the contamination of cells cultured in the

plates were controlled microscopically. Typical signs of contamination are changes in color or clouding of cell medium and changes in cell shape.

11. The medium was discarded from the plates.
12. The cells were incubated with sinapic acid at the concentrations between 15.625 – 20000 µM for an additional 18 hours.
13. At the end of the incubation, the solution was discarded from the plates and 200 µl of NR standard solution at 37^0C was added to all wells. The plates were incubated for additional 3 hours in the incubator.
14. At the end of the incubation, the solution was carefully discarded and plate was washed three times with pre-warmed (37^0C) sterile PBS under the safety cabinet. For washing procedure, 200 µL of the PBS was added to each well by a multichannel pipette and then discarded. Each plate firmly was tapped on absorbent paper cloth to remove any remaining liquid from the wells.
15. 200 µL of NR fixative solution was added to each well.
16. The plates, wrapped in foil to be kept in the dark, were placed on shaker for 20 minutes at 600 rpm.
17. Plate lids were removed just before placing each plate on the plate reader.
18. The absorbance of the samples was recorded at 540 nm wavelength spectrofotometrically.
19. The data were expressed as average values obtained from 8 wells for each concentration. The results were plotted as percent of control (assuming data obtained from the absence of sinapic acid as 100%) and expressed as the mean percentage of cell viability. The cytotoxicity experiments were repeated three times.

2.4.3. Solutions of MTT Assay

- MTT stock solution (5 mg/ml)

5 mg of MTT is dissolved in 1 ml of PBS. Solution must be sterilized using Millipore filter after adding MTT, kept away from light. It can be stored at 4^0C up to 4 days.

- MTT reagent

40 ml of dimethylformamide (DMF), 2 ml of glacial acetic acid, and 58 ml of distilled water are mixed. Then 16 g of sodium dodecyl sulfate (SDS) are added to this solution. The pH is adjusted to 4.7.

2.4.4. Procedure of MTT Assay

1. V79 and HeLa cells were seeded and cultured as mentioned at 2.4.2.Procedure of NRU Assay/ section 1-11.
2. After 24 hours incubation, the cells in a 96-well plate were incubated with sinapic acid at the concentrations between 15.625 – 20000 µM at 37 ^{0}C in a humidified atmosphere of 5% CO_2 in air for 24 hours.
3. At the end of the incubation, the solution was discarded from the plates, 180 µl of culture medium and 20 µl of MTT stock solution were added to all wells. The cells were incubated with MTT (0.5 mg/ml) at 37^{0}C for additional 4 hours.
4. At the end of the incubation, the solution was carefully discarded. Each plate firmly was tapped on absorbent paper cloth to remove any remaining liquid from the wells.
5. 200 µL of MTT reagent was added to each well.
6. The plates, wrapped in foil to be kept in the dark, were placed on shaker for 15 minutes at 600 rpm.
7. Plate lids were removed just before placing each plate on the plate reader.
8. The absorbance of the samples was recorded at 570 nm wavelength spectrofotometrically.
9. The data were expressed as average values obtained from 8 wells for each concentration. The results were plotted as percent of control (assuming data obtained from the absence of sinapic acid as 100%) and expressed as the mean percentage of cell viability. The cytotoxicity experiments were repeated three times.

2.5. Genotoxicity Assays

2.5.1. Assessment of DNA Damage by the Alkaline Single Cell Gel Electrophoresis (Comet Assay)

a) Solutions of Comet Assay

- **Electrophoresis Buffer**

1750 ml cold distilled water, 52.8 ml NaOH 10 N, 8.8 ml 200mM EDTA are mixed properly (pH 13.0). It should be freshly prepared on the day of the experiment.

- **EDTA 200 mM**

14.89 g EDTA salt is dissolved in 200 ml distilled water. The pH is adjusted to 10 and the solution is kept at 4^0C.

- **Ethidium Bromide (EtBr) Staining Solution**

To prepare the stock staining solution, 10 mg EtBr is dissolved in 50 ml distilled water. Then 1 ml of this solution is taken and the volume is adjusted to 10 ml with distilled water. Both solutions are kept at room temperature.

- **Low Melting Agarose (LMA) 0.65% Solution**

162.5 mg LMA is dissolved in 25 ml PBS in microwave avoiding the boiling of agarose. After the agarose is dissolved, the solution has been divided into small amounts and kept in refrigerator.

- **Stock Lysing Solution**

146.1 g NaCl, 37.2 g EDTA, 1.2 g Tris are dissolved in 500 ml of distilled water. After adjusting pH to 10 by 10 g of NaOH, 10 g of N-Lauryl sarcosinate sodium salt is added. The contents should be properly mixed, and then the volume is adjusted to 1000 ml with distilled water. The stock lysing is kept at room temperature.

- **Lysing Solution**

178 ml of stock lysing solution is mixed with 20 ml of DMSO and 2 ml of Triton-X100

. It should be freshly prepared 1 hour before the experiment and should be kept at 4^0C.

- **Neutralization Buffer**

48.5 mg Tris is dissolved in 750 ml distilled water and the pH is set to 7.5. The volume is adjusted to 1000 ml distilled water and the buffer is stored at the room temperature.

- **Normal Melting Agarose (NMA) 1% Solution**

1g NMA is dissolved in 100 ml PBS in hot water avoiding the agarose to boil. The solution is kept in small volumes in refrigerator.

- **Sodium Hydroxide (NaOH) 10 N**

200 g NaOH is dissolved in 500 ml distilled water and the solution is kept at room temperature. The shelf life of the 10 N NaOH stock solution is limited to 2 weeks.

- **Phosphate Buffered Saline (PBS) Buffer**

One tablet of PBS is dissolved in 200 ml distilled water and then the buffer is sterilized and stored at 4 ^{0}C.

b) Procedure of Comet Assay for Human Lymphocytes

The basic alkaline technique was described by Singh et al. (142) as further described by Collins et al. (143) was followed.

- **Blood Sampling and Lymphocyte Preparations**

1. A 5 ml heparinized (50 units/mol sodium heparin) whole peripheral blood sample was taken by venipuncture from the individual (with no smoking and alcohol drinking habit).

2. The whole blood was diluted with 5 ml PBS and gently layered on 2.5 ml Histopaque (lymphorep) by a Pasteur pipette in a centrifuge tube.
3. The mixture was centrifuged for 20 minutes at 2400 rpm.
4. The cells on to the Ficoll layer in the interphase were carefully taken by a Pasteur pipette after centrifugation and transferred to the tube.
5. PBS was added to the lymphocytes and mixed.
6. The cells were centrifuged at 2400 rpm for 10 minutes.
7. The supernatant was poured off and the lymphocytes were washed with PBS again in the same manner.
8. The 1 x 10^4 cells in 50 µl were treated with the increasing concentrations of sinapic acid (50, 150, 300, 500, 1000, 2000, and 5000 µM) for 60 min at 37°C in a humidified atmosphere of 5% CO_2 in air for the assessment of DNA damage.
9. After the pretreatment of sinapic acid for 60 min, the lymphocytes were centrifugated and washed with PBS. Then oxidative damage was induced by replacing the medium with PBS containing 50 µM H_2O_2 and then incubating for 5 min on ice to assess the antigenotoxicity of sinapic acid.
10. Then the lymphocytes were centrifugated and washed with PBS for removing the H_2O_2 solution.
11. A negative control sample (PBS with 1% DMSO) and positive control 50 µM H_2O_2 were also included in the experiments. In the experiments, the percentages of DMSO did not exceed 1% by volume.
12. After centrifugation at 3000 rpm, supernatant was discarded and 50 µl of the cells were suspended in 100 µl of 0.65% LMA.

- **Slide Preparation**

1. The suspensions were embedded on the slides pre-coated with a layer of 1% NMA using a coverslip.
2. The slides were allowed to solidify on ice for 5 min.
3. After removal of the coverslip, the slides were immersed in cold freshly made lysing solution for a minimum for 1 hour at 4 ^{0}C.

- **Electrophoresis**

1. The slides were removed from the lysing solution, drained and were left in the electrophoresis solution for 20 min at 4 ^{0}C to allow unwinding of the DNA and expression of alkali-labile damage.
2. Electrophoresis was conducted also at a low temperature (4 ^{0}C) for 20 min using 25 V and adjusting the current to 300 mA by raising or lowering the buffer level.
3. The slides were neutralized by washing 3 times in 0.4 M Tris-HCl (pH 7.5) for 5 min at room temperature.
4. After neutralization, the slides were incubated in 50%, 75%, and 99% of alcohol for 5 min, successively.
5. The dried microscope slides were stained with ethidium bromide (EtBr 20 µg/ml in distilled water, 35 µl / slide), covered with a coverslip prior to analysis.

- **Slide Scoring**

1. For visualization of the DNA damage, the slides were examined with a Leica fluorescence microscope under green light.

2. The microscope was connected to a charge-coupled device camera and a personal computer-based analysis system (Comet Analysis Software, version 3.0, Kinetic Imaging Ltd., Liverpool, UK) to determine the extent of DNA damage after electrophoretic migration of the DNA fragments in the agarose gel.

3. In order to visualize DNA damage, slides were examined at 40x (Figure 2.1). One-hundred cells from two replicate slides were assayed for each experiment. Results were expressed as DNA tail intensity, and tail moment, tail migration. The experiments were repeated three times.

Figure 2.1. The Comet images of human lymphocytes under the fluorescent microscope

c) Procedure of Comet Assay for V79 (Chinese Hamster Fibroblast) Cell Line

- **Preparations of V79 Cell Line**

All the procedure must be carried out in the laminar flow safety cabinet. The safety cabinet has been thoroughly cleaned and all equipments have been wiped down with 70% ethanol before use.

1. V79 cells were seeded and cultured as mentioned at section 2.4.2.Procedure of NRU Assay/ section 1-11.
2. After 24 hours incubation, the cells in a 96-well plate were treated with the increasing concentrations of sinapic acid (50, 150, 300, 500, 1000, 2000, and 5000 µM) at 37 ^{0}C in a humidified atmosphere of 5% CO_2 in air for 60 min.
3. To assess the antigenotoxicity of sinapic acid, after the pretreatment of sinapic acid, oxidative damage was induced by replacing the medium with PBS containing 50 µM H_2O_2 and then incubating for 5 min .
4. Then the cells were washed with sterile PBS for removing the H_2O_2 solution.
5. The negative control sample (PBS with 1% DMSO) and positive control 50 µM H_2O_2 were included in the experiments. The concentration of DMSO did not exceed 1 %.
6. After incubation time for the treatments, the medium was aspirated.
7. 200 µl of sterilized PBS at 37^{0}C was added to the plate to wash the cells, and PBS

was aspirated. Then 75 µl of trypsin-EDTA (10X) at 37^0C was added to the flask for trypsinization of the cells. The cells were incubated for 5 min at 37^0C in the incubator.

8. After incubation, cells were detached from the plate and checked under the microscope to ensure all cells being detached.
9. 150 µl of culture medium with fetal calf serum at 37 ^{0}C was added to the plate to stop the reaction.
10. Cells were centrifuged for 5 min at 1200 rpm. The supernatant was discarded by aspiration.
11. The cells were washed with 500 µl of the culture medium.
12. Cells were centrifuged for 5 min at 1200 rpm. The supernatant was discarded by aspiration.
13. 10000 cells/50 µl of the medium were suspended in 100 µl of 0.65% LMA.

- **Slide Preparation, electrophoresis, and slide scoring**

The processes of slide preparation, electrophoresis, and slide scoring were performed as mentioned at the section of "Procedure of Comet Assay for Human Lymphocytes".

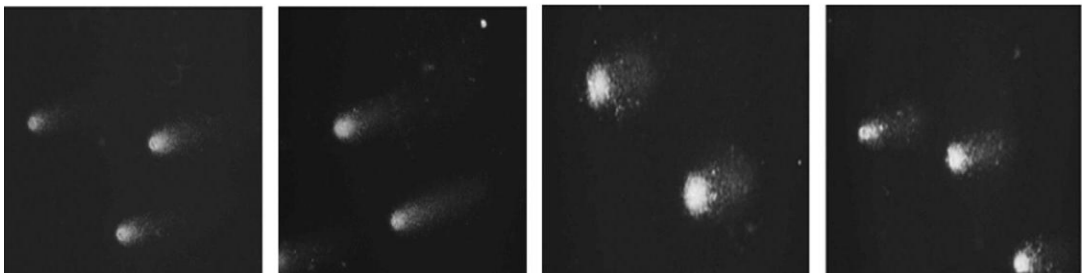

Figure 2.2. The Comet images of V79 cell lines under the fluorescent microscope

2.5.2. Cytokinesis-Blocked Micronucleus Assay (CBMN)

The presence of MN in a binuclated cell using the protocol of Fenech (138) was determined with minor modifications. Human periferal blood cultures were used for the CBMN test.

a) Solutions of Micronucleus Assay

- **Culture Medium for MN**

100 ml of RPMI 1640, 20 ml of fetal calf serum, 2.5 ml PHA-M, 2 ml L-glutamine and 2 ml penicillin-streptomycin are mixed. This culture medium should be kept in -20^{0}C.

- **Mitomycin C (MMC) Stock Solution 5 mg/ml**

5 mg of MMC powder is dissolved in 1 ml of sterile distilled water. This solution should be kept in -20^{0}C.

- **Mitomycin C Standart Solution 0.5 mg/ml**

100 µl of mitomycin C stock solution (5mg/ml) is mixed with 900µl of PBS.

- **Hypotonic Solution**

1.397 g KCl is dissolved in 250 ml dissolved water. This solution should be stored at -20 ^{0}C.

- **Fixation Solution**

100 ml acetic acid is mixed with 300 ml methanol. (3:1 is the ratio of methanol: acetic acid). This solution should be stored at -20^{0}C.

- **Nitric Acid 65%**

68.75 ml nitric acid is dissolved in 1000 ml distilled water. This solution is stored at dark.

- **Cytochalasin-B (Cyt-B)**

1 mg Cyt-B is dissolved in 1.475 ml sterile DMSO. This solution should be stored at -20 ^{0}C.

- **0.01 M Phosphate Buffer Solution**

0.435 g K_2HPO_4 is dissolved in 250 ml distilled water. 0.340 g KH_2PO_4 is dissolved in 250 ml distilled water. 50 ml of 0.01 M K_2HPO_4 and 50 ml of 0.01 M KH_2PO_4 are mixed. Then pH is adjusted to 6.8.

- **Giemsa Dye 5%**

5 ml of Giemsa dye are mixed with 95 ml of 0.01 M phosphate buffer solution. It is filtered using whatmann paper.

b) Procedure of CBMN Assay for Human Lymphocytes

- **Sampling for Human Lymphocytes**

1. A 5-ml heparinized (0.2 ml heparine per 5 ml whole blood) peripheral blood sample was taken by venipuncture from the individual (with no smoking and no alcohol drinking habits).
2. 0.5 ml blood was added to the tubes containing 5 ml cultured medium for MN. Medium should be warmed to 37^{0}C.
3. Tubes were incubated at 37^{0}C in a humidified atmosphere containing 5% CO_2 for 24h.
4. After 24 hours (one cell cycle), different concentrations of sinapic acid (50, 150, 300, 1000, 2000, and 5000 μM) were added to the tubes alone and also in combination with mitomycin C (0.2 μg/ml).

5. In all sets of experiments, an untreated negative control (1% DMSO) as well as a positive control (mitomycin C (0.2 μg/ml)) were also run simultaneously. All tubes were incubated for another 24 h.
6. After another 20 hours (total 44 hours), 50 μL Cyt-B (an inhibitor of the mitotic spindle) was added to all tubes.
7. 28 hours after adding Cyt-B, the tubes were centrifuged at 1000 rpm for 10 minutes. The supernatant was discarded and 0.5-0.7 ml of cell suspension was retained in the tubes and suspended. 5 ml of ice-cold hypotonic KCl solution were added to the tubes very slowly. Tubes were kept at 4^0C for 5 minutes.
8. Tubes were centrifuged at 1000 rpm for another 10 minutes. The supernatant was discarded and 5 ml of fixation solution was added to the tubes very slowly.
9. Tubes were kept at 4^0C for 15 minutes. This step should be repeated twice.
10. After the last centrifuge, the supernatant was discarded and the 0.5-0.7 ml of cell suspension was retained in the tubes and was homogenized by Pasteur pipette.
11. The fixed cells were dropped onto slides previously cleaned with nitric acid. The slides were allowed to air dry.
12. Then the slides were stained in 5% Giemsa for 10-15 min and coded for different concentrations.
13. The experiment was repeated three times.

- **Examination of slides and assessment of MN for Human Lymphocytes**

After staining, the slides were air-dried and binucleated cells were counted. Slides were examined at 40 x objectives using a light microscope. A score should be obtained for slides from each duplicate culture. The number of cells scored should be determined depending on the level of change in the MN index that the experiment is intended to detect and the expected standard deviation of the estimate. For each slide, the following information should be obtained. The number of micronuclei (MNi) in at least 1000 binucleate (BN) cells should be scored (Figure 2.3).

The criteria for scoring MNi in BN cells were detailed below:

- The distribution of BN cells with zero, one or more MNi; the number of MNi in

a single binucleated cell normally ranges from 0 to 3 in lymphocytes of healthy individuals but can be greater than 3 on occasion depending on chemical exposure and age.

- The frequency of micronucleated BN cells in at least 1000 BN cells and the proportion of mononucleated, binucleated, tri-nucleated and tetra-nucleated cells per 500 cells were scored (Figure 2.4).

The Nuclear Division Index can be derived as follows;

- [1 x N1] + [2 x N2] + [3 x (N3+N4)] / N, where N1-N4 represent the number of cells with 1-4 nuclei, respectively, and N is the total number of cells scored.
- The cytokinesis-blocked cells that may be scored for MN frequency should have the following characteristics: (a) The cells should be binucleated; (b) The two nuclei in a binucleated cell should have intact nuclear membranes and be situated within the same cytoplasmic boundary.

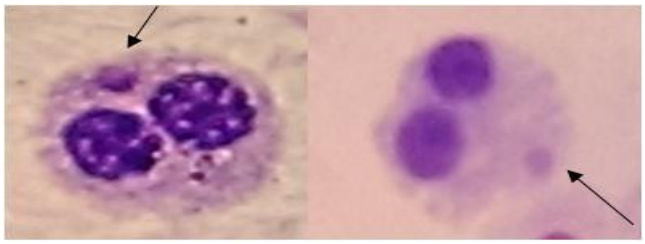

Figure 2.3. The MN images of human lymphocytes

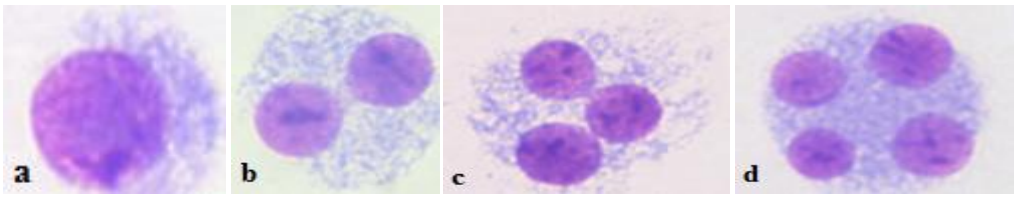

Figure 2.4. The images of mononucleated (a), binucleated (b), tri-nucleated (c), and tetra-nucleated (d) lymphocytes under microscope

c) Procedure of CBMN Assay for V79 Cells

- **Sampling for V79 Cells**

All the procedure must be carried out in the laminar flow safety cabinet. The safety cabinet has been thoroughly cleaned and all equipments have been wiped down with 70% ethanol before use.

1. V79 cells were seeded and cultured as mentioned at section 2.4.2.Procedure of NRU Assay/ section 1-8.
2. For the experiment, 10000 cells/well in 2000 µL culture medium were seeded into a 6-well plate. The plate was controlled under a phase contrast microscope.
3. The plate was incubated at 37^0C for 24 hours in a humidified atmosphere of 5% CO_2 in air.
4. After 24 hours, the viability and also the contamination of cells cultured in the plates are controlled microscopically. Typical signs of contamination are changes in color or clouding of cell medium and changes in cell shape.
5. The medium was discarded from the plate.
6. The cells were treated with the increasing concentrations of sinapic acid (50, 150, 300, 500, 1000, 2000, and 5000 µM) and with/without mitomycin C (0.2 µg/ml) for 24 h.
7. In all sets of experiments, an untreated negative control (1% DMSO) as well as a positive control (mitomycin C (0.2 µg/ml)) was also run simultaneously. The concentration of DMSO did not exceed 1 %.
8. 24 hours after treatment, the medium was aspirated from the plates. The cells were washed twice with 5 mL ice-cold PBS, pH 7.4, and Cyt-B was added to fresh complete culture medium to obtain a final concentration of 3 µg/ml. The cells were incubated for an additional 20h to block cytokinesis and yield binucleated cells.
9. After incubation, the medium was aspirated from the plates.
10. 2 ml of sterilized 37^0C warm PBS was added to the plate to wash the cells, and PBS was aspirated. Then 750 µl of 37^0C warm trypsin-EDTA (10X) was added to the flask for trypsinization of the cells. It was incubated for 5 min in the incubator.

11. After incubation, cells were detached from the plate and checked under the microscope to ensure all cells being detached
12. 1500 μl of 37 ^{0}C culture medium with fetal calf serum was added to the plate to stop the reaction.
13. The cell suspensions were transferred to the sterile tubes.
14. The tubes were centrifuged at 1000 rpm for 10 minutes.
15. The supernatant was discarded and 0.5-0.7 ml of cell suspension was retained in the tubes and suspended. 5 ml of ice-cold hypotonic KCl solution were added to the tubes very slowly. Tubes were kept at 4^{0}C for 5 minutes.
16. Tubes were centrifuged at 1000 rpm for another 10 minutes. The supernatant was discarded and 5 ml of fixation solution was added to the tubes very slowly.
17. Tubes were kept at 4^{0}C for 15 minutes. This step should be repeated twice.
18. After the last centrifuge, the supernatant was discarded and the 0.5-0.7 ml of cell suspension was retained in the tubes and was homogenized by Pasteur pipette.
19. The fixed cells were dropped onto slides previously cleaned with nitric acid.
20. The slides were allowed to air dry.
21. Then the slides were stained in 5% Giemsa for 10-15 min and coded for different concentrations.
22. The experiment was repeated independently three times.

- **Examination of slides and assessment of MN for V79 Cells**

The examination and assessment of MN for V79 cells was performed as mentioned above for the lymphocytes (Figure 2.5).

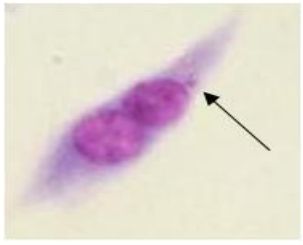

Figure 2.5. The MN images of V79 cell lines

2.6. Statistical Analysis

For alkaline comet assay, statistical analysis was performed by SPSS for windows 11.5 computer program. The results were expressed as the mean ± standard deviation. Difference between the means of data are compared by the one way variance analysis (ANOVA) test and the post hoc analysis of group differences by least significant difference (LSD) test. *P* value of less than 0.05 was considered as statistically significant. For statistical analysis of micronucleus assay results, the z-test was applied for the percentage of MN and CBPI. The results were given as the mean ± standard error.

3. RESULTS

3.1. Determination of the Cytotoxicity of Sinapic Acid

3.1.1. Determination of the Cytotoxicity of Sinapic Acid by Neutral Red Uptake (NRU) Assay

The cytotoxic effects of different concentrations of sinapic acid on V79 cells as measured by the NRU assay have been shown in Figure 3.1. V79 cell line was chosen because of its high sensitivity to various chemicals, high cloning efficiency, and excellent properties in colony formation.

According to the results, compared to the same concentrations of untreated cells (control group); sinapic acid was found to have cytotoxic effects in concentrations more than 2000 µM. A concentration dependent toxicity was observed in V79 cells after 18 hours exposure to sinapic acid.

The concentrations up to 500 µM had no significant effect on V79 cell viability during 18 hours exposure but at concentrations higher than 1000 µM, the cell viability decreased below 50 %. IC_{50} value of sinapic acid in V79 cell line was found to be 1860 µM.

The cytotoxic effects of the different concentrations of sinapic acid on HeLa cells as measured by the NRU assay has been shown in Figure 3.2

According to the results, compared to the same concentrations of untreated cells (control group); sinapic acid was found to have cytotoxic effects in concentrations more than 5000 µM. A concentration dependent toxicity was observed in HeLa cells after 18 hours exposure to sinapic acid.

The concentrations up to 1000 µM had no effect on HeLa cell viability during 18 hours exposure but at concentrations higher than 5000 µM, the cell viability decreased below 50 %. IC_{50} value of sinapic acid in HeLa cell line was found to be 7248 µM.

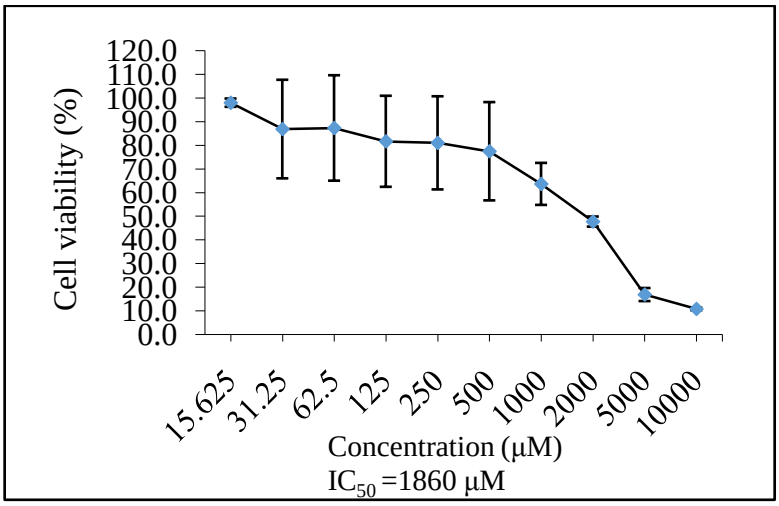

Figure 3.1. Effects of sinapic acid on V79 cells viability in NRU assay*

*Results were given as the mean ± standard deviation. The results were expressed as percentage of cell growth inhibition from three independent experiments. Cell viability was plotted as percent of control (assuming data obtained from absence of sinapic acid as 100%). NRU: neutral red uptake

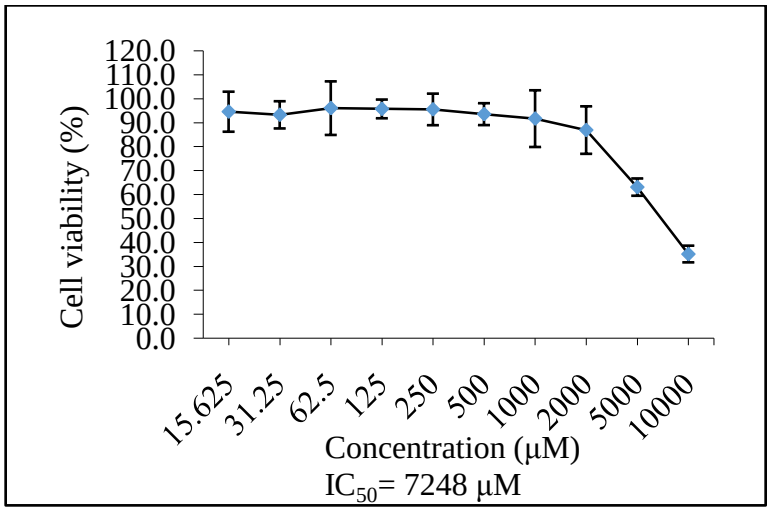

Figure 3.2 Effects of sinapic acid on HeLa cells viability in NRU assay*

*Results were given as the mean ± standard deviation. The results were expressed as percentage of cell growth inhibition from three independent experiments. Cell viability was plotted as percent of control (assuming data obtained from absence of sinapic acid as 100%). NRU: neutral red uptake

3.1.2. Determination of the Cytotoxicity of Sinapic Acid by MTT Assay

The cytotoxic effects of the different concentrations of sinapic acid as measured by the MTT assay have been shown in Figure 3.3.

According to the results, compared to the same concentrations of untreated cells (control group); sinapic acid was found to have cytotoxic effects in concentrations more than 5000 µM. A concentration dependent toxicity was observed in V79 cells after 24 hours exposure to sinapic acid.

The concentrations up to 2000 µM had no effect on V79 cell viability during 24 hours exposure but at concentrations higher than 5000 µM, the cell viability decreased below 50 %. IC_{50} value of sinapic acid in V79 cell line was found to be 8658 µM.

The cytotoxic effects of the different concentrations of sinapic acid in HeLa cells as measured by the MTT assay have been shown in Figure 3.4

According to the results, compared to the same concentrations of untreated cells (control group); sinapic acid was found to have cytotoxic effects in concentrations more than 5000 µM. A concentration dependent toxicity was observed in HeLa cells after 24 hours exposure to sinapic acid.

The concentrations up to 5000 µM had no effect on HeLa cell viability during 24 hours exposure but at concentrations higher than 10000 µM, the cell viability decreased below 50 %. IC_{50} value of sinapic acid in HeLa cell line was found to be 12033 µM (12 mM).

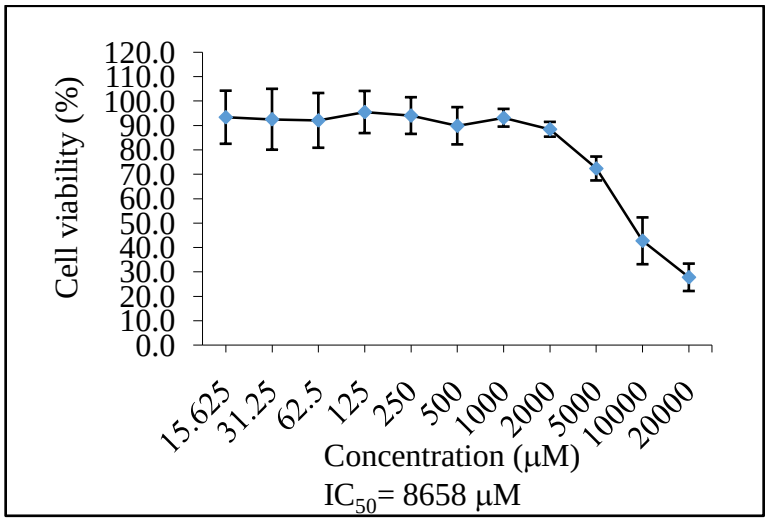

Figure 3.3. Effects of sinapic acid on V79 cells viability in MTT assay*

*Results were given as the mean ± standard deviation. The results were expressed as percentage of cell growth inhibition from three independent experiments. Cell viability was plotted as percent of control (assuming data obtained from absence of sinapic acid as 100%).

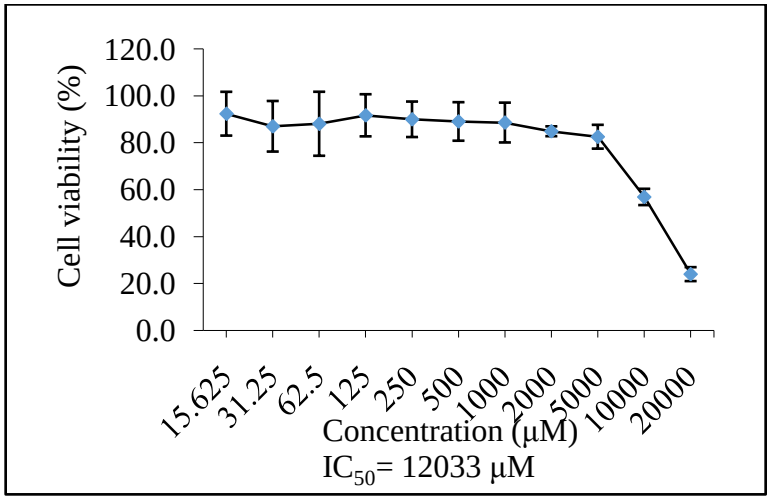

Figure 3.4. Effects of sinapic acid on HeLa cells viability in MTT assay*

*Results were given as the mean ± standard deviation. The results were expressed as percentage of cell growth inhibition from three independent experiments. Cell viability was plotted as percent of control (assuming data obtained from absence of sinapic acid as 100%).

3.2. Assessment of the Genotoxicity and Antigenotoxicity of Sinapic Acid by Comet Assay

3.2.1. Assessment of the Genotoxicity and Antigenotoxicity of Sinapic Acid in the Human Lymphocytes by Comet Assay

The results for the assessment of the genotoxicity and the antigenotoxicity of sinapic acid in human lymphocytes using alkaline comet assay was shown in Figure 3.5. and Table 3.1. According to the three independent experiments, no significant increase in DNA strand breakage expressed as DNA tail intensity, DNA tail moment, and DNA tail migration was observed at low sinapic acid concentrations of 50 µM, 150 µM, and 300 µM, whereas at the concentrations of 500 µM, 1000 µM, and 2000 µM of sinapic acid alone caused an increase in the DNA damage when compared to negative control (p<0.05). At the concentrations between 50 µM and 2000 µM, sinapic acid seemed to decrease H_2O_2-induced DNA damage (p<0.05), although it was found that at the highest concentration of 5000 µM, sinapic acid did not decrease H_2O_2-induced DNA damage in the lymphocytes.

3.2.2. Assessment of the Genotoxicity and Antigenotoxicity of Sinapic Acid in V79 Cell Line by Comet Assay

The results for the assessment of the genotoxicity and antigenotoxicity of sinapic acid in V79 cells using alkaline comet assay were shown in Figure 3.6. and Table 3.2. according to the three independent experiments. As seen in Figure 3.6.(A), no significant increase in DNA strand breakage expressed as DNA tail intensity was observed below 1000 µM, however, at the concentrations of 1000 µM, 2000 µM, and 5000 µM of sinapic acid alone caused an increase in DNA damage compared to negative control in a dose dependent manner (p<0.05).

As seen in Figure 3.6.(B), no significant increase in DNA strand breakage expressed as DNA tail moment was observed below 2000 µM, however, at the concentrations of 2000 µM and 5000 µM of sinapic acid alone caused an increase in DNA damage compared to negative control in a dose dependent manner (p<0.05).

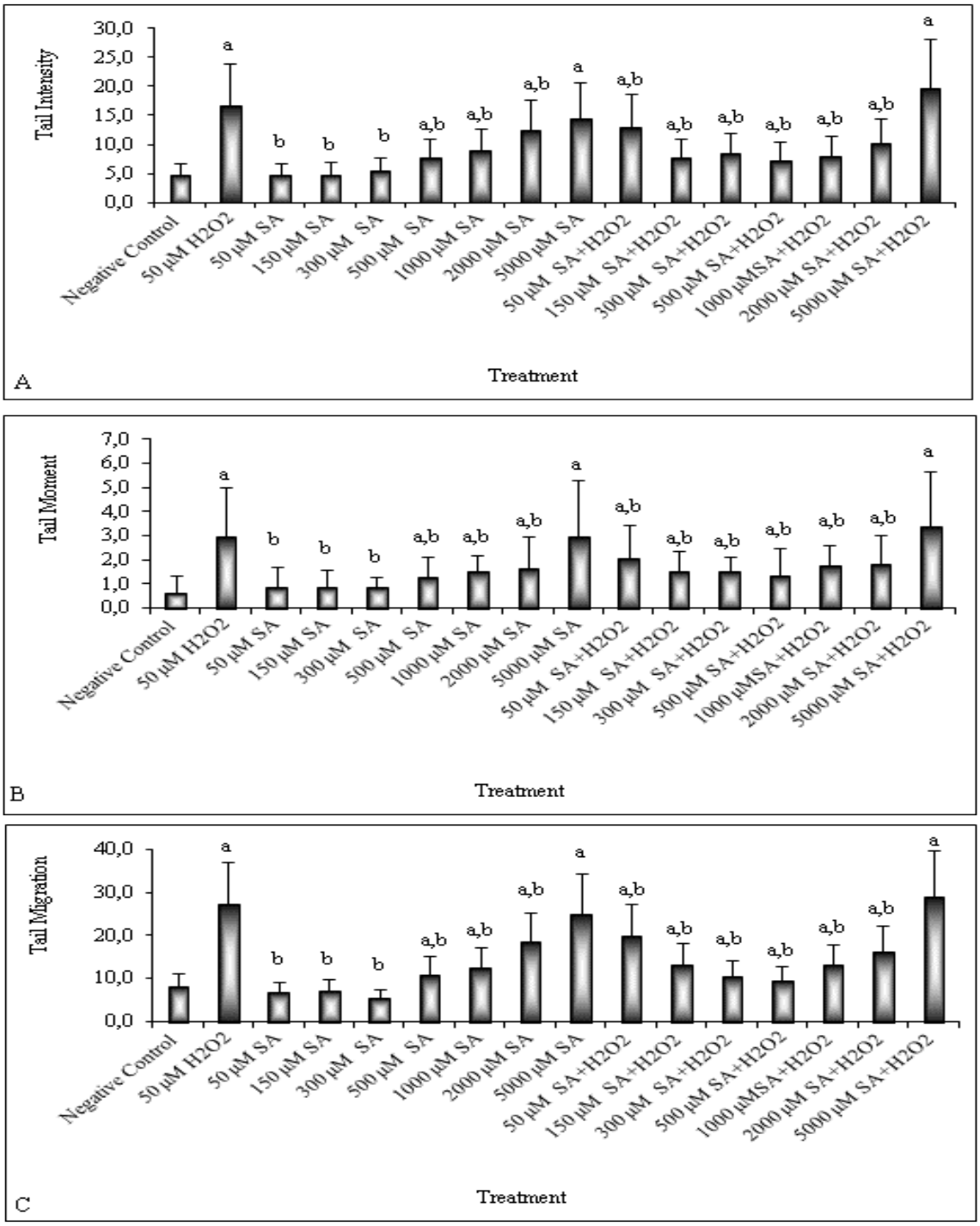

Figure 3.5. DNA damage in the lymphocytes expressed as DNA tail intensity (A), DNA tail moment (B), and DNA tail migration (C). SA= sinapic acid. Values were given as mean ± standard deviation, n=3. [a]$p<0.05$, statistically different from negative control. [b]$p<0.05$, statistically different from positive control (50 µM H_2O_2).

Table 3.1. The assessment of genotoxicity and antigenotoxicity of sinapic acid with/without H_2O_2 in human lymphocytes using Comet assay*

		DNA Tail Intensity	DNA Tail Moment	DNA Tail Migration
1	**Negative control**	4.68±2.00	0.66±0.69	8.19±3.00
2	**50 µM H_2O_2**	16.86±7.20 [a]	3.00±2.02 [a]	27.20±9.97 [a]
3	**50 µM SA**	4.71±2.01 [b]	0.90±0.80 [b]	6.84±2.51 [b]
4	**150 µM SA**	4.87±2.08 [b]	0.86±0.73 [b]	7.23±2.65 [b]
5	**300 µM SA**	5.48±2.34 [b]	0.90±0.42 [b]	5.59±2.05 [a,b]
6	**500 µM SA**	7.79±3.33 [a,b]	1.27±0.84 [a,b]	11.06±4.05 [a,b]
7	**1000 µM SA**	8.89±3.80 [a,b]	1.52±0.70 [a,b]	12.75±4.67 [a,b]
8	**2000 µM SA**	12.49±5.34 [a,b]	1.68±1.27 [a,b]	18.51±6.79 [a,b]
9	**5000 µM SA**	14.49±6.19 [a]	2.98±2.35 [a]	25.05±9.18 [a]
10	**50 µM SA + H_2O_2**	13.10±5.60 [a,b]	2.10±1.37 [a,b]	19.96±7.32 [a,b]
11	**150 µM SA + H_2O_2**	7.69±3.28 [a,b]	1.51±0.87 [a,b]	13.42±4.92 [a,b]
12	**300 µM SA + H_2O_2**	8.50±3.63 [a,b]	1.54±0.62 [a,b]	10.55±3.87 [a,b]
13	**500 µM SA + H_2O_2**	7.37±3.15 [a,b]	1.38±1.09 [a,b]	9.41±3.45 [a,b]
14	**1000 µM SA + H_2O_2**	8.13±3.47 [a,b]	1.79±0,81 [a,b]	13.24±4.85 [a,b]
15	**2000 µM SA + H_2O_2**	10.21±4,36 [a,b]	1.83±1.20 [a,b]	16,36±6.00 [a,b]
16	**5000 µM SA + H_2O_2**	19.74±8.43 [a]	3.41±2.29 [a]	29.13±10.68 [a]

*DNA damage expressed as DNA tail intensity, DNA tail moment, and DNA tail migration. SA= sinapic acid. Values were given as mean ± standard deviation.

[a] $p<0.05$, statistically different from negative control (1% DMSO).

[b] $p<0.05$, statistically different from positive control (50 µM H_2O_2).

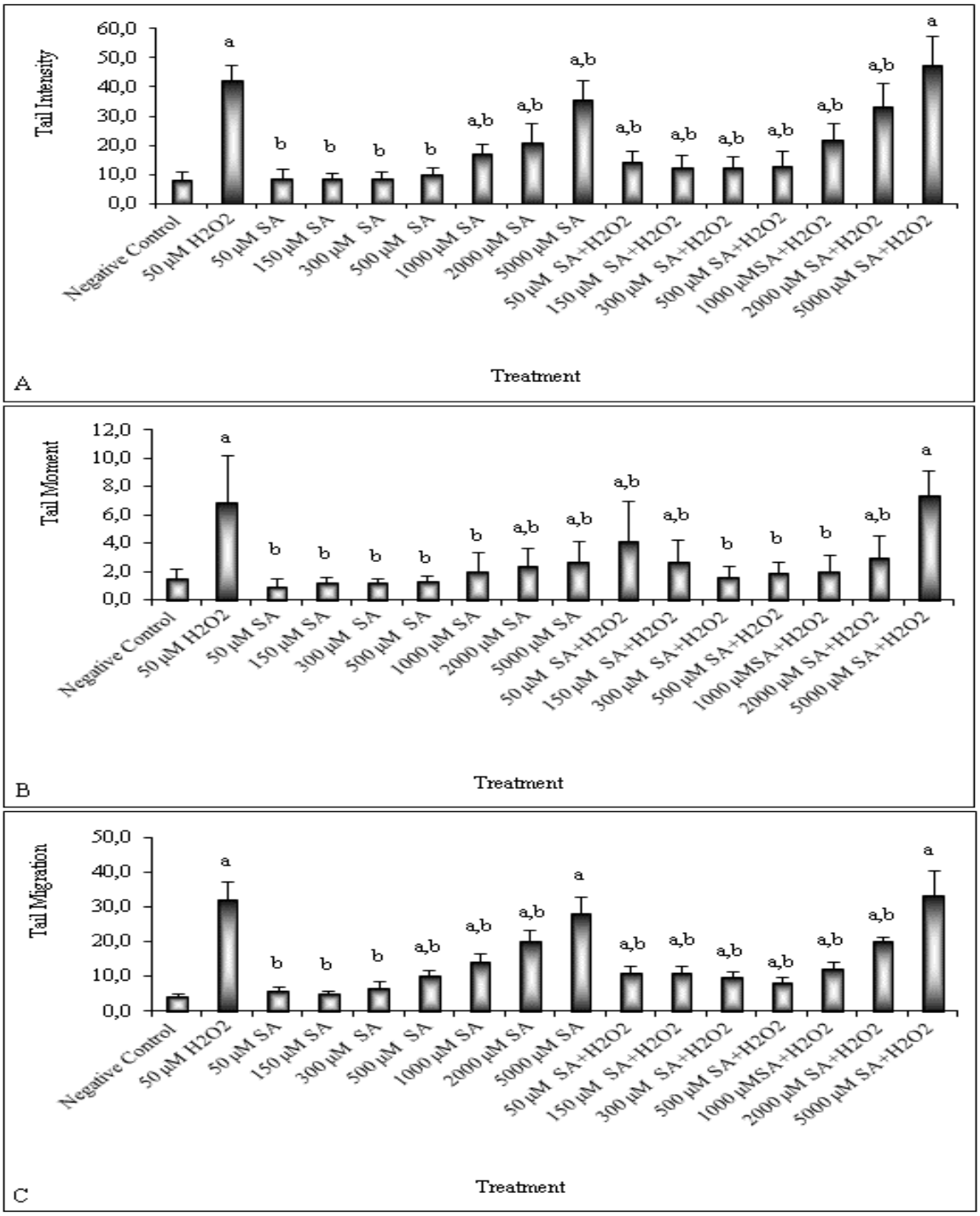

Figure 3.6. DNA damage in V79 cells expressed as DNA tail intensity (A), DNA tail moment (B), and DNA tail migration (C). SA= sinapic acid. Values were given as mean ± standard deviation, n=3. [a]$p<0.05$, statistically different from negative control. [b]$p<0.05$, statistically different from positive control (50 µM H_2O_2).

Table 3.2. The assessment of genotoxicity and antigenotoxicity of sinapic acid with/without H_2O_2 in V79 cells using Comet assay*

		DNA Tail Intensity	DNA Tail Moment	DNA Tail Migration
1	**Negative control**	8.19±3.00	1.50±0.69	4.43±0.73
2	**50 µM H_2O_2**	42.18±5.61 [a]	6.89±3.32 [a]	32.08±5.36 [a]
3	**50 µM SA**	8.76±3.07 [b]	0.97±0.54 [b]	6.07±1.01 [b]
4	**150 µM SA**	8.83±1.62 [b]	1.19±0.42 [b]	5.04±0.84 [b]
5	**300 µM SA**	8.68±2.33 [b]	1.20±0.28 [b]	6.49±2.02 [b]
6	**500 µM SA**	9.83±2.44 [b]	1.35±0.35 [b]	10.46±1.24 [a,b]
7	**1000 µM SA**	17.37±2.97 [a,b]	2.00±1.37 [b]	14.18±2.37 [a,b]
8	**2000 µM SA**	21.08±6.44 [a,b]	2.39±1.24 [a,b]	20.10±3.36 [a,b]
9	**5000 µM SA**	35.60±7.00 [a,b]	2.72±1.45 [a,b]	28.29±4.72 [a]
10	**50 µM SA + H_2O_2**	14.25±3.83 [a,b]	4.17±2.80 [a,b]	11.21±1.87 [a,b]
11	**150 µM SA + H_2O_2**	12.48±4.34 [a,b]	2.72±1.56 [a,b]	11.25±1.88 [a,b]
12	**300 µM SA + H_2O_2**	12.58±3.52 [a,b]	1.65±0.70 [b]	9.77±1.63 [a,b]
13	**500 µM SA + H_2O_2**	12.85±5.05 [a,b]	1.88±0.81 [b]	8.18±1.58 [a,b]
14	**1000 µM SA + H_2O_2**	21.85±5.72 [a,b]	2,05±1,10 [b]	12,39±1,94 [a,b]
15	**2000 µM SA + H_2O_2**	33.17±8.09 [a,b]	3,03±1.56 [a,b]	20.05±1.47 [a,b]
16	**5000 µM SA + H_2O_2**	47.78±9.75 [a]	7.39±1.79 [a]	33.40±7.22 [a]

*DNA damage expressed as DNA tail intensity, DNA tail moment, and DNA tail migration. SA= sinapic acid. Values were given as mean ± standard deviation.

[a] $p<0.05$, statistically different from negative control (1% DMSO).

[b] $p<0.05$, statistically different from positive control (50 µM H_2O_2).

As seen in Figure 3.6.(C), no significant increase in DNA strand breakage expressed as DNA tail migration was observed below 500 µM, however, above 500 µM of sinapic acid alone caused an increase in DNA damage compared to negative control in a dose dependent manner (p<0.05).

At the concentrations between 50 µM and 2000 µM, sinapic acid seemed to decrease H_2O_2-induced DNA damage expressed as DNA tail intensity, DNA tail moment, and DNA tail migration (p<0.05). However, it was found that at the highest concentration of 5000 µM, sinapic acid did not decrease H_2O_2-induced DNA damage in V79 cells.

3.3. Assessment of the Genotoxicity and Antigenotoxicity of Sinapic Acid by CBMN Assay

3.3.1. Assessment of the Genotoxicity and Antigenotoxicity of Sinapic Acid by CBMN Assay in Human Lymphocytes

The results of the experiments for the genotoxicity and antigenotoxicity testing by MN formation in human lymphocytes were given in Table 3.3. The lymphocytes treated with different concentrations of sinapic acid (50-2000 µM) alone caused no clastogenic effects when compared to negative control. However, sinapic acid at the concentration of 5000 µM, significantly increased the MN frequencies when compared to negative control (p<0.05).

The treatment of sinapic acid with 0.2 µg/ml MMC revealed a reduction in the MN frequencies at the concentrations of 50-2000 µM sinapic acid when compared to the samples treated with MMC (p<0.05). However, 5000 µM of sinapic acid did not decrease the MN frequencies when compared to the samples treated with MMC. The effects of sinapic acid with/without MMC on nuclear division showed that there was no significant difference in the cell division at the treatment of sinapic acid and MMC when compared to negative control (Table 3.3).

3.3.2. Assessment of the Genotoxicity and Antigenotoxicity of Sinapic Acid by CBMN Assay in V79 Cell Line

The results of the experiments for the clastogenicity and anticlastogenicity testing by MN formation in V79 cells were shown in Table 3.4. The cells treated with different concentrations of sinapic acid (50-1000 µM) alone caused no clastogenic effects when compared to negative control. However, sinapic acid at the concentration of 2000 µM and 5000µM, significantly increased the MN frequencies when compared to negative control ($p<0.05$).

The treatment of sinapic acid with 0.2 µg/ml MMC revealed reduction in the MN frequencies at the concentrations of 50-2000 µM sinapic acid when compared to the samples treated with MMC ($p<0.05$). However at the highest concentration of sinapic acid (5000 µM), the MN frequencies increased significantly when compared to the samples treated with MMC ($p<0.05$). There were no significant difference in the cell division at the treatments of sinapic acid and MMC when compared to negative control (Table 3.3).

Table 3.3. Micronucleus frequencies and the cytokinesis block proliferation index of sinapic acid with/without mitomycin C in human lymphocytes.

Treatment group	**BN cells scored**	**Distributions of BN cells according to the no of MN**			**MN/10^3 cells**	**MN (%) ± SE**	**CBPI±SE**
Negative control	3000	1	1	4	3.33	0.33 ±0.13	1.87±0.33
0.2 µg/ml MMC	3000	86	74	79	79.67*	7.97 ±0.35	1.73±0.26
50 µM SA	3000	2	3	4	3.00#	0.30 ±0.06	1.66±0.26
150 µM SA	3000	3	3	5	3.67#	0.37±0.07	1.62±0.23
300 µM SA	3000	3	2	6	3.67#	0.37 ±0.12	2.00±0.48
500 µM SA	3000	4	1	4	3.13#	0.31 ±0.11	1.63±0.20
1000 µM SA	3000	4	2	5	3.61#	0.36 ±0.09	1.64±0.22
2000 µM SA	3000	5	2	7	4.67#	0.47 ±0.15	1.68±0.29
5000 µM SA	3000	8	7	10	8.33*#	0.83 ±0.09	1.74±0.38
50 µM SA + MMC	3000	45	33	36	38.00*#	3.80 ±0.36	1.66±0.28
150 µM SA + MMC	3000	33	24	26	27.67*#	2.77 ±0.27	1.83±0.32
300 µM SA + MMC	3000	33	15	18	22.00*#	2.20 ±0.56	2.04±0.30
500 µM SA + MMC	3000	21	24	16	20.33*#	2.03 ±0.23	1.72±0.23
1000 µM SA + MMC	3000	33	36	30	33.00*#	3.30 ±0.17	1.64±0.25
2000 µM SA + MMC	3000	39	27	24	30.00*#	3.00 ±0.46	1.71±0.23
5000 µM SA + MMC	3000	84	72	64	73.33*	7.33 ±0.58	1.52±0.25

* $p<0.05$, significantly different from negative control (z-test).

# $p<0.05$, significantly different from MMC treatment (z-test).

SA= sinapic acid; MMC=mitomycin C; Negative control (1% DMSO); BN= binucleated; MN= micronucleus; SE= standard error; CBPI= cytokinesis blocked proliferation index.

Table 3.4. Micronucleus frequencies and the cytokinesis block proliferation index of sinapic acid with/without MMC in V79 cells.

Treatment group	**BN cells scored**	**Distributions of BN cells according to the no of MN**			**MN/10^3 cells**	**MN (%) ± SE**	**CBPI±SE**
Negative control	3000	3	5	4	4.00	0.40±0.06	1.95 ± 0.04
0.2 µg/ml MMC	3000	66	73	71	70.00*	7.00±0.09	1.86 ± 0.40
50 µM SA	3000	4	4	5	4.33#	0.43±0.03	1.92 ± 0.13
150 µM SA	3000	2	6	3	3.67#	0.63±0.12	1.94 ± 0.05
300 µM SA	3000	3	5	5	4.33#	0.43±0.07	1.67 ± 0.06
500 µM SA	3000	4	4	3	3.67#	0.37±0.03	1.78 ± 0.07
1000 µM SA	3000	3	2	6	3.67#	0.37±0.12	1.89 ± 0.08
2000 µM SA	3000	5	5	9	6.33*#	0.63±0.13	1.81 ± 0.09
5000 µM SA	3000	12	9	11	10.67*#	1.07±0.09	1.56 ± 0.10
50 µM SA + MMC	3000	24	21	25	23.33*#	2.33±0.12	1.66 ± 0.11
150 µM SA + MMC	3000	18	28	10	18.67*#	1.87±0.52	1.77 ± 0.12
300 µM SA + MMC	3000	12	14	25	17.00*#	1.70±0.40	1.90 ± 0.24
500 µM SA + MMC	3000	24	35	10	23.00*#	2.30±0.72	1.73 ± 0.13
1000 µM SA +MMC	3000	18	35	20	24.33*#	2.43±0.54	1.78 ± 0.13
2000 µM SA +MMC	3000	32	38	41	37.00*#	3.70±0.26	1.74 ± 0.13
5000 µM SA +MMC	3000	87	81	97	88.33*#	8.83±0.47	1.58 ± 0.23

* $p<0.05$, significantly different from negative control (z-test).

# $p<0.05$, significantly different from MMC treatment (z-test).

SA= sinapic acid; MMC=mitomycin C; Negative control (1%DMSO); BN= binucleated; MN= micronucleus; SE= standard error.

4. DISCUSSION

Phenolic compounds are a group of key plant metabolites found abundantly in fruit and vegetables. Because of their antioxidant properties, they play an important role in the prevention of various degenerative disorders or diseases related to oxidative damage.

The antioxidant activity of phenolic compounds depends on the structure, in particular the number and the positions of the hydroxyl groups and the nature of substitutions on the aromatic rings. Phenolic compounds are present as conjugates with mono- and polysaccharides, linked to one or more of the phenolic groups, and may also occur as functional derivatives such as esters and methyl esters.

Sinapic acid is a small naturally occurring hydroxycinnamic acid derivative. It is a phenolic compound and a member of the phenylpropanoid family, which are assumed as therapeutically beneficial and generally not toxic. Sinapic acid is widespread in the plant kingdom (fruits, vegetables, cereal grains, oilseed crops, and some spices and medicinal plants) and is common in the human diet. Derivatives of sinapic acid are characteristic compounds of the *Brassicaceae* family. Sinapic acid has been suggested to show antioxidant, antimicrobial, anti-inflammatory, anticancer, and anxiolytic activity.

On other hand, there are very few studies related to the cytotoxicity and genotoxicity of sinapic acid. The cytotoxicity of sinapic acid has been studied in human neuroblastoma cells (SH-SY5Y) by using MTT assay. The cells were incubated with different concentrations of sinapic acid (0, 1, 10, and 100μg/ml) for 24 h. The results showed that sinapic acid did not induce cytotoxic effect on SH-SY5Y cells at the concentrations studied (117).

In an another study by Jingli et al. (125), the cytotoxic effect of sinapic acid was also tested on SH-SY5Y at different concentrations for 24 h by using the 4-[3-(4-Iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1, 3-benzene disulfonate (WST-1) assay. Sinapic acid did not affect the viability of human neuroblastoma cells within the concentration range used (0–20.0 μM), the EC_{50} of sinapic acid in this study was found to be 1.957 ± 0.23 μM. In this study, sinapic acid exhibited moderate protection against

H_2O_2-induced cell death in dose dependent manner.

Antiproliferative and apoptotic effects of sinapic acid was examined in human breast cancer cells (T47D). Sinapic acid was found to be only partial inhibitor to cell growth, decreasing cell proliferation with IC_{50} of 7×10^{-11} M. It was also reported that the inhibitory effect and pro-apoptotic effect of sinapic acid on tumoral proliferation might be due to its direct interaction with the aryl hydrocarbon receptor, the nitric oxide synthase inhibition and its pro-apoptotic effect (100).

In our study, the cytotoxicity of sinapic acid using the NRU and MTT assay in V79 and HeLa cells which are high sensitivity to various chemicals, high cloning efficiency, and excellent properties in colony formation was investigated. Our results demonstrated that, at the concentrations up to 500 µM, sinapic acid had no effect on V79 cell viability during 18 hours exposure but at concentrations higher than 1000 µM, the cell viability decreased below 50 % and IC_{50} value of sinapic acid in V79 cell line was found to be 1860 µM by NRU assay.

The cytotoxicity of sinapic acid was also determined on HeLa cell line by NRU assay. At the concentrations up to 1000 µM, sinapic acid had no effect on HeLa cell viability during 18 hours exposure but at concentrations higher than 5000 µM, the cell viability decreased below 50 %. IC_{50} value of sinapic acid in HeLa cell line was found to be 7248 µM. We also investigated the *in vitro* cytotoxicity of sinapic acid by the MTT assay test in V79 and HeLa cells. Our results demonstrated that sinapic acid was found to have cytotoxic effects in concentrations above 2000 µM in V79 cells after 24 hours exposure to sinapic acid.

At the concentrations up to 2000 µM, sinapic acid had no effect on V79 cell viability during 24 hours exposure but at the concentrations higher than 5000 µM, the cell viability decreased below 50 %. IC_{50} value of sinapic acid in V79 cell line was found to be 8658 µM by MTT assay.

The cytotoxicity of sinapic acid was also determined on HeLa cell line by MTT assay. At the concentrations up to 5000 µM, sinapic acid had no effect on HeLa cell viability during 24 hours exposure but at concentrations higher than 10000 µM, the cell viability decreased below 50 %. IC_{50} value of sinapic acid in HeLa cell line was found to

be 12033 µM.

In conclusion, the IC_{50} values of sinapic acid in V79 cells were found as 1860 µM and 8658 µM by NRU and MTT assays, respectively. In addition, the IC_{50} values of sinapic acid in HeLa cells were observed as 7248 µM and 12033 µM by NRU and MTT assays, respectively. Since both cytotoxicity assays are well validated and well-accepted assays in many laboratories, it is difficult to explain the differences of the IC_{50} values of sinapic acid in both assays. However, it is clear from two assays that sinapic acid is not cytotoxic to V79 and HeLa cells below 2000 µM.

There are also very few studies regarding the genotoxicity of sinapic acid. In our study, the genotoxicity potential of sinapic acid was investigated by the alkaline comet assay and the cytokinesis-blocked micronucleus assay (CBMN).

The comet assay was performed to investigate whether sinapic acid provides protection against H_2O_2 induced DNA damage in human peripheral lymphocytes and V79 cells. H_2O_2 is a highly reactive oxygen species and can induce damage to proteins, nucleic acids, and cell membranes. It is known to cause oxidative DNA damage primarily through the hydroxyl radical which result from Fenton reaction. H_2O_2 has been reported to cause DNA damage in the form of chromosomal aberrations, single- and double- strand breaks (144).

The genotoxic effect of sinapic acid was investigated in human peripheral lymphocyte by alkaline comet assay in the range of 50-5000 µM concentrations. No significant increase in DNA strand breakage was observed at low concentrations of 50-300 µM, whereas 500-2000 µM of sinapic acid alone caused an increase in the DNA damage when compared to negative control. However, the concentrations between 50 µM and 2000 µM, sinapic acid seemed to decrease H_2O_2-induced DNA damage, although it was found that at the highest concentration of 5000 µM, sinapic acid did not decrease H_2O_2-induced DNA damage in the lymphocytes.

We also investigated the genotoxicity and antigenotoxicity of sinapic acid in V79 cells using alkaline comet assay. No significant increase in DNA strand breakage was observed below 1000 µM, however, at the concentration of 1000-5000 µM of sinapic acid alone caused an increase in DNA damage compared to negative control in a dose

dependent manner. At the concentrations between 50 µM and 2000 µM, sinapic acid seemed to decrease H_2O_2-induced DNA damage. However, it was found that at the highest concentration of 5000 µM, sinapic acid did not decrease H_2O_2-induced DNA damage in V79 cells.

In an *in vitro* study, the antigenotoxic effects of sinapic acid were investigated on the growth of human adenocarcinoma colon cells (HT-29) by using comet assay. The result showed that sinapic acid has found to exert antigenotoxic effect on human adenocarcinoma colon cells with EC_{50} (3.7± 3.1 µmol/L) (126).

There are many studies on the phenolic compounds for their genotoxic/antigenotoxic effects, however there are not enough data on sinapic acid. Maistro et al. (145), examined the genotoxic and clastogenic potential of three phenolic compounds such as caffeic, cinnamic and ferulic acids, using the comet and micronucleus assays *in vitro* in rat hepatoma tissue cells (HTCs). Three different concentrations (50, 500, and 1500 µM) of these phenolic acids were tested on the HTCs for 24 h. The caffeic, cinnamic and ferulic acids were not found to be genotoxic by the comet assay, however, in the micronucleus test an increase in the frequency of micronucleated cells for the three compounds were observed, indicating that these substances have clastogenic effects in HTC (145).

Some phenolic compounds were found to be genotoxic but the mechanisms involved in this process are not fully understood. For example, the induction of chromosomal aberrations by phenol, catechol and pyrogallol in V79 cells at different pH values (6.0, 7.4, and 8.0) were observed. Catechol and pyrogallol showed clear clastogenic effect in a pH-dependent way. Taken together, it was suggested that, despite the structural similarity between the different molecules studied, the mechanisms of genotoxicity of these molecules could be considerably different. The existence of several mechanisms of genotoxicity, partially shared by this class of compounds, could explain the synergistic effects observed between these compounds in several genotoxicity test systems (146).

In this study, the clastogenicity and anticlastogenicity of sinapic acid was also investigated in human lymphocytes by micronucleus assay. The cells treated with

different concentrations of sinapic acid (50-2000 µM) alone caused no clastogenic effects when compared to negative control. However, sinapic acid at the concentration of 5000 µM, significantly increased the MN frequencies when compared to negative control.

The treatment of sinapic acid with 0.2 µg/ml MMC is revealed a reduction in the MN frequencies at the concentrations of 50-2000 µM sinapic acid when compared to the lymphocyte treated with MMC. However, 5000 µM of sinapic acid did not decrease the MN frequencies when compared to the samples treated with MMC. The effects of sinapic acid with/without MMC on nuclear division showed that there was no significant difference in the cell division at the treatment of sinapic acid and MMC when compared to negative control. However, up to now, there is not enough data about the cytotoxicity and genotoxicity of sinapic acid; the available data are limited and contrary.

In conclusion, the results of this study suggest that sinapic acid might have cytotoxic effects in a dose dependent manner in different cell lines. However, sinapic acid in the concentrations used below the IC_{50} values showed no significant genotoxic effects. At the concentrations below IC_{50} values of sinapic acid did not induce DNA damage both in lymphocytes and V79 cells. Sinapic acid seemed to decrease the oxidative DNA damage induced by H_2O_2. The results of MN assay also showed that sinapic acid might protect against MMC-induced genotoxicity. As our study is composed only an *in vitro* experiment, further *in vivo* animal studies are required to understand the genotoxic and antigenotoxic properties of sinapic acid in detail.

REFERENCES

1. Tarko, T., Duda-Chodak, A.,Zając, N. (2013) Digestion and absorption of phenolic compounds assessed by in vitro simulation methods. A review. *Roczniki Państwowego Zakładu Higieny*, 64 (2), 79-84.
2. Benavente-García, O., Castillo, J., Marin, F.R., Ortuño, A.,Del Río, J.A. (1997) Uses and properties of citrus flavonoids. *Journal of Agricultural and Food Chemistry*, 45 (12), 4505-4515.
3. Manach, C., Mazur, A.,Scalbert, A. (2005) Polyphenols and prevention of cardiovascular diseases. *Current Opinion in Lipidology*, 16 (1), 77-84.
4. Middleton, E., Kandaswami, C.,Theoharides, T.C. (2000) The effects of plant flavonoids on mammalian cells: implications for inflammation, heart disease, and cancer. *Pharmacological Reviews*, 52 (4), 673-751.
5. Bravo, L. (1998) Polyphenols: chemistry, dietary sources, metabolism, and nutritional significance. *Nutrition Reviews*, 56 (11), 317-333.
6. Balasundram, N., Sundram, K.,Samman, S. (2006) Phenolic compounds in plants and agri-industrial by-products: Antioxidant activity, occurrence, and potential uses. *Food Chemistry*, 99 (1), 191-203.
7. Harborne, J.B. (1989). Methods in plant biochemistry. Volume 1. Plant phenolics. London: Academic Press Ltd.
8. Harborne, J.J.B., Baxter, H.,Moss, G.P. (1999). Phytochemical dictionary: a handbook of bioactive compounds from plants. London: CRC Press.
9. Hertog, M.G., Feskens, E.J., Kromhout, D., Hollman, P.,Katan, M. (1993) Dietary antioxidant flavonoids and risk of coronary heart disease: the Zutphen Elderly Study. *The Lancet*, 342 (8878), 1007-1011.
10. Spanos, G.A., Wrolstad, R.E.,Heatherbell, D.A. (1990) Influence of processing and storage on the phenolic composition of apple juice. *Journal of Agricultural and Food Chemistry*, 38 (7), 1572-1579.
11. Ferguson, L.R. (2001) Role of plant polyphenols in genomic stability. *Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis*, 475 (1), 89-

111.

12. Naczk, M., Oickle, D., Pink, D.,Shahidi, F. (1996) Protein precipitating capacity of crude canola tannins: effect of pH, tannin, and protein concentrations. *Journal of Agricultural and Food Chemistry*, 44 (8), 2144-2148.
13. Reddy, M.B.,Cook, J.D. (1991) Assessment of dietary determinants of nonheme-iron absorption in humans and rats. *The American Journal of Clinical Nutrition*, 54 (4), 723-728.
14. Özeker, E. (1999) Phenolic compounds and their importance. *Anadolu, J. of AARI*, 9 (2), 114-124.
15. Rice-Evans, C.A., Miller, N.J.,Paganga, G. (1996) Structure-antioxidant activity relationships of flavonoids and phenolic acids. *Free Radical Biology and Medicine*, 20 (7), 933-956.
16. Back, K. (2001) Hydroxycinnamic acid amides and their possible utilization for enhancing agronomic traits. *The Plant Pathology Journal*, 17 (3), 123-127.
17. Mattila, P., Pihlava, J.-m.,Hellström, J. (2005) Contents of phenolic acids, alkyl- and alkenylresorcinols, and avenanthramides in commercial grain products. *Journal of Agricultural and Food Chemistry*, 53 (21), 8290-8295.
18. Mattila, P.,Hellström, J. (2007) Phenolic acids in potatoes, vegetables, and some of their products. *Journal of Food Composition and Analysis*, 20 (3), 152-160.
19. Mattila, P., Hellström, J.,Törrönen, R. (2006) Phenolic acids in berries, fruits, and beverages. *Journal of Agricultural and Food Chemistry*, 54 (19), 7193-7199.
20. Fukuda, H.,Komamine, A. (1982) Lignin synthesis and its related enzymes as markers of tracheary-element differentiation in single cells isolated from the mesophyll of Zinnia elegans. *Planta*, 155 (5), 423-430.
21. Herrmann, K.,Nagel, C.W. (1989) Occurrence and content of hydroxycinnamic and hydroxybenzoic acid compounds in foods. *Critical Reviews in Food Science and Nutrition*, 28 (4), 315-347.
22. Adom, K.K.,Liu, R.H. (2002) Antioxidant activity of grains. *Journal of Agricultural and Food Chemistry*, 50 (21), 6182-6187.
23. Clifford, M.N. (1999) Chlorogenic acids and other cinnamates–nature, occurrence

and dietary burden. *Journal of the Science of Food and Agriculture*, 79 (3), 362-372.

24. Mattila, P.,Kumpulainen, J. (2002) Determination of free and total phenolic acids in plant-derived foods by HPLC with diode-array detection. *Journal of Agricultural and Food Chemistry*, 50 (13), 3660-3667.
25. El-Seedi, H.R., El-Said, A.M., Khalifa, S.A., Göransson, U., Bohlin, L., Borg-Karlson, A.-K. ve diğerleri. (2012) Biosynthesis, natural sources, dietary intake, pharmacokinetic properties, and biological activities of hydroxycinnamic acids. *Journal of Agricultural and Food Chemistry*, 60 (44), 10877-10895.
26. Mølgaard, P.,Ravn, H. (1988) Evolutionary aspects of caffeoyl ester distribution in dicotyledons. *Phytochemistry*, 27 (8), 2411-2421.
27. Cremin, P., Kasim-Karakas, S.,Waterhouse, A.L. (2001) LC/ES-MS detection of hydroxycinnamates in human plasma and urine. *Journal of Agricultural and Food Chemistry*, 49 (4), 1747-1750.
28. Manach, C., Scalbert, A., Morand, C., Rémésy, C.,Jiménez, L. (2004) Polyphenols: food sources and bioavailability. *The American Journal of Clinical Nutrition*, 79 (5), 727-747.
29. Podsędek, A. (2007) Natural antioxidants and antioxidant capacity of Brassica vegetables: A review. *LWT-Food Science and Technology*, 40 (1), 1-11.
30. Kroon, P.A.,Williamson, G. (1999) Hydroxycinnamates in plants and food: current and future perspectives. *Journal of the Science of Food and Agriculture*, 79 (3), 355-361.
31. Germano, M., d'Angelo, V., Biasini, T., Sanogo, R., De Pasquale, R.,Catania, S. (2006) Evaluation of the antioxidant properties and bioavailability of free and bound phenolic acids from Trichilia emetica Vahl. *Journal of Ethnopharmacology*, 105 (3), 368-373.
32. Nićiforović, N.,Abramovič, H. (2014) Sinapic Acid and Its Derivatives: Natural Sources and Bioactivity. *Comprehensive Reviews in Food Science and Food Safety*, 13 (1), 34-51.
33. Vuorela, S., Meyer, A.S.,Heinonen, M. (2004) Impact of isolation method on the

antioxidant activity of rapeseed meal phenolics. *Journal of Agricultural and Food Chemistry*, 52 (26), 8202-8207.

34. Thiyam, U., Stöckmann, H.,Schwarz, K. (2006) Antioxidant activity of rapeseed phenolics and their interactions with tocopherols during lipid oxidation. *Journal of the American Oil Chemists' Society*, 83 (6), 523-528.
35. Tzagoloff, A. (1963) Metabolism of sinapine in mustard plants. I. Degradation of sinapine into sinapic acid & choline. *Plant Physiology*, 38 (2), 202.
36. Chapple, C., Vogt, T., Ellis, B.E.,Somerville, C.R. (1992) An Arabidopsis mutant defective in the general phenylpropanoid pathway. *The Plant Cell Online*, 4 (11), 1413-1424.
37. Shirley, A.M.,Chapple, C. (2003) Biochemical characterization of sinapoylglucose: choline sinapoyltransferase, a serine carboxypeptidase-like protein that functions as an acyltransferase in plant secondary metabolism. *Journal of Biological Chemistry*, 278 (22), 19870-19877.
38. Murray, C., Norris, D.J.,Bawendi, M.G. (1993) Synthesis and characterization of nearly monodisperse CdE (E= sulfur, selenium, tellurium) semiconductor nanocrystallites. *Journal of the American Chemical Society*, 115 (19), 8706-8715.
39. Cai, R., Arntfield, S.,Charlton, J. (1999) Structural changes of sinapic acid and sinapine bisulfate during autoclaving with respect to the development of colored substances. *Journal of the American Oil Chemists' Society*, 76 (4), 433-441.
40. Cartea, M.E., Francisco, M., Soengas, P.,Velasco, P. (2010) Phenolic compounds in Brassica vegetables. *Molecules*, 16 (1), 251-280.
41. Wang, Y.-C., Chuang, Y.-C.,Ku, Y.-H. (2007) Quantitation of bioactive compounds in citrus fruits cultivated in Taiwan. *Food Chemistry*, 102 (4), 1163-1171.
42. Russell, W.R., Labat, A., Scobbie, L., Duncan, G.J.,Duthie, G.G. (2009) Phenolic acid content of fruits commonly consumed and locally produced in Scotland. *Food Chemistry*, 115 (1), 100-104.
43. Zuo, Y., Wang, C.,Zhan, J. (2002) Separation, characterization, and quantitation

of benzoic and phenolic antioxidants in American cranberry fruit by GC-MS. *Journal of Agricultural and Food Chemistry*, 50 (13), 3789-3794.

44. Andreasen, M.F., Christensen, L.P., Meyer, A.S.,Hansen, Å. (2000) Content of Phenolic Acids and Ferulic Acid Dehydrodimers in 17 Rye (Secale c ereale L.) Varieties. *Journal of Agricultural and Food Chemistry*, 48 (7), 2837-2842.
45. Bunzel, M., Allerdings, E., Sinwell, V., Ralph, J.,Steinhart, H. (2002) Cell wall hydroxycinnamates in wild rice (Zizania aquatica L.) insoluble dietary fibre. *European Food Research and Technology*, 214 (6), 482-488.
46. Hudson, E.A., Dinh, P.A., Kokubun, T., Simmonds, M.S.,Gescher, A. (2000) Characterization of potentially chemopreventive phenols in extracts of brown rice that inhibit the growth of human breast and colon cancer cells. *Cancer Epidemiology Biomarkers & Prevention*, 9 (11), 1163-1170.
47. Hatcher, D.,Kruger, J. (1997) Simple Phenolic Acids in Flours Prepared from Canadian Wheat: Relationship to Ash Content, Color, and Polyphenol Oxidase Activity 1. *Cereal Chemistry*, 74 (3), 337-343.
48. Takaya, Y., Kondo, Y., Furukawa, T.,Niwa, M. (2003) Antioxidant constituents of radish sprout (Kaiware-daikon), Raphanus sativus L. *Journal of Agricultural and Food Chemistry*, 51 (27), 8061-8066.
49. Vuorela, S., Meyer, A.S.,Heinonen, M. (2003) Quantitative analysis of the main phenolics in rapeseed meal and oils processed differently using enzymatic hydrolysis and HPLC. *European Food Research and Technology*, 217 (6), 517-523.
50. Ayaz, F.A., Hayırlıoglu-Ayaz, S., Alpay-Karaoglu, S., Grúz, J., Valentová, K., Ulrichová, J. ve diğerleri. (2008) Phenolic acid contents of kale (Brassica oleraceae L. var. acephala DC.) extracts and their antioxidant and antibacterial activities. *Food Chemistry*, 107 (1), 19-25.
51. Wu, X.,Prior, R.L. (2005) Identification and characterization of anthocyanins by high-performance liquid chromatography-electrospray ionization-tandem mass spectrometry in common foods in the United States: vegetables, nuts, and grains. *Journal of Agricultural and Food Chemistry*, 53 (8), 3101-3113.

52. Thiyam, U., Stöckmann, H., Zum Felde, T.,Schwarz, K. (2006) Antioxidative effect of the main sinapic acid derivatives from rapeseed and mustard oil by-products. *European Journal of Lipid Science and Technology*, 108 (3), 239-248.

53. Bennett, R.N., Rosa, E.A., Mellon, F.A.,Kroon, P.A. (2006) Ontogenic profiling of glucosinolates, flavonoids, and other secondary metabolites in Eruca sativa (salad rocket), Diplotaxis erucoides (wall rocket), Diplotaxis tenuifolia (wild rocket), and Bunias orientalis (Turkish rocket). *Journal of Agricultural and Food Chemistry*, 54 (11), 4005-4015.

54. Kern, S.M., Bennett, R.N., Mellon, F.A., Kroon, P.A.,Garcia-Conesa, M.-T. (2003) Absorption of hydroxycinnamates in humans after high-bran cereal consumption. *Journal of Agricultural and Food Chemistry*, 51 (20), 6050-6055.

55. Zhang, K.,Zuo, Y. (2004) GC-MS determination of flavonoids and phenolic and benzoic acids in human plasma after consumption of cranberry juice. *Journal of Agricultural and Food Chemistry*, 52 (2), 222-227.

56. Hemery, Y.M., Anson, N.M., Havenaar, R., Haenen, G.R., Noort, M.W.,Rouau, X. (2010) Dry-fractionation of wheat bran increases the bioaccessibility of phenolic acids in breads made from processed bran fractions. *Food Research International*, 43 (5), 1429-1438.

57. Nardini, M., Forte, M., Vrhovsek, U., Mattivi, F., Viola, R.,Scaccini, C. (2009) White wine phenolics are absorbed and extensively metabolized in humans. *Journal of Agricultural and Food Chemistry*, 57 (7), 2711-2718.

58. Shivashankara, K.,Acharya, S. (2010) Bioavailability of Dietary Polyphenols and the Cardiovascular Diseases. *Open Nutraceuticals Journal*, 3.

59. Couteau, D., McCartney, A., Gibson, G., Williamson, G.,Faulds, C. (2001) Isolation and characterization of human colonic bacteria able to hydrolyse chlorogenic acid. *Journal of Applied Microbiology*, 90 (6), 873-881.

60. Chesson, A., Provan, G.J., Russell, W.R., Scobbie, L., Richardson, A.J.,Stewart, C. (1999) Hydroxycinnamic acids in the digestive tract of livestock and humans. *Journal of the Science of Food and Agriculture*, 79 (3), 373-378.

61. Wolffram, S., Weber, T., Grenacher, B.,Scharrer, E. (1995) A Na+-Dependent Mechanism Is Involved in Mucosal uptake of Cinnamic Acid across the Jejunal Brush Border in Rats.
62. Ader, P., Grenacher, B., Langguth, P., Scharrer, E.,Wolffram, S. (1996) Cinnamate uptake by rat small intestine: transport kinetics and transepithelial transfer. *Experimental Physiology*, 81 (6), 943-955.
63. Clifford, M.N. (2000) Chlorogenic acids and other cinnamates–nature, occurrence, dietary burden, absorption and metabolism. *Journal of the Science of Food and Agriculture*, 80 (7), 1033-1043.
64. Konishi, Y.,Kobayashi, S. (2005) Transepithelial transport of rosmarinic acid in intestinal Caco-2 cell monolayers. *Biosci Biotechnol Biochem*, 69 (3), 583-591.
65. Zhao, Z.,Moghadasian, M.H. (2010) Bioavailability of hydroxycinnamates: a brief review of in vivo and in vitro studies. *Phytochemistry Reviews*, 9 (1), 133-145.
66. Jin, X.-L., Wei, X., Qi, F.-M., Yu, S.-S., Zhou, B.,Bai, S. (2012) Characterization of hydroxycinnamic acid derivatives binding to bovine serum albumin. *Organic and Biomolecular Chemistry*, 10 (17), 3424-3431.
67. Smyk, B. (2003) Fluorescence study of sinapic acid interaction with bovine serum albumin and egg albumin. *Journal of Fluorescence*, 13 (4), 349-356.
68. Cos, P., Rajan, P., Vedernikova, I., Calomme, M., Pieters, L., Vlietinck, A.J. ve diğerleri. (2002) In vitro antioxidant profile of phenolic acid derivatives. *Free Radical Research*, 36 (6), 711-716.
69. Marques, V.,Farah, A. (2009) Chlorogenic acids and related compounds in medicinal plants and infusions. *Food Chemistry*, 113 (4), 1370-1376.
70. Siquet, C., Paiva-Martins, F., Lima, J.L., Reis, S.,Borges, F. (2006) Antioxidant profile of dihydroxy-and trihydroxyphenolic acids-A structure-activity relationship study. *Free Radical Research*, 40 (4), 433-442.
71. Teixeira, S., Siquet, C., Alves, C., Boal, I., Marques, M.P., Borges, F. ve diğerleri. (2005) Structure–property studies on the antioxidant activity of flavonoids present in diet. *Free Radical Biology and Medicine*, 39 (8), 1099-

1108.

72. Nenadis, N., Lazaridou, O.,Tsimidou, M.Z. (2007) Use of reference compounds in antioxidant activity assessment. *Journal of Agricultural and Food Chemistry*, 55 (14), 5452-5460.

73. Firuzi, O., Giansanti, L., Vento, R., Seibert, C., Petrucci, R., Marrosu, G. ve diğerleri. (2003) Hypochlorite scavenging activity of hydroxycinnamic acids evaluated by a rapid microplate method based on the measurement of chloramines. *Journal of Pharmacy and Pharmacology*, 55 (7), 1021-1027.

74. Niwa, T., Doi, U., Kato, Y.,Osawa, T. (1999) Inhibitory mechanism of sinapinic acid against peroxynitrite-mediated tyrosine nitration of protein in vitro. *FEBS letters*, 459 (1), 43-46.

75. Zou, Y., Kim, A.R., Kim, J.E., Choi, J.S.,Chung, H.Y. (2002) Peroxynitrite scavenging activity of sinapic acid (3, 5-dimethoxy-4-hydroxycinnamic acid) isolated from Brassica juncea. *Journal of Agricultural and Food Chemistry*, 50 (21), 5884-5890.

76. Kikuzaki, H., Hisamoto, M., Hirose, K., Akiyama, K.,Taniguchi, H. (2002) Antioxidant properties of ferulic acid and its related compounds. *Journal of Agricultural and Food Chemistry*, 50 (7), 2161-2168.

77. Nenadis, N.,Tsimidou, M. (2002) Observations on the estimation of scavenging activity of phenolic compounds using rapid 1, 1-diphenyl-2-picrylhydrazyl (DPPH•) tests. *Journal of the American Oil Chemists' Society*, 79 (12), 1191-1195.

78. Jin, X., Yang, R., Shang, Y., Dai, F., Qian, Y., Cheng, L. ve diğerleri. (2010) Oxidative coupling of cinnamic acid derivatives and their radical-scavenging activities. *Chinese Science Bulletin*, 55 (25), 2885-2890.

79. Wanasundara, U.N., Amarowicz, R.,Shahidi, F. (1995) Partial characterization of natural antioxidants in canola meal. *Food Research International*, 28 (6), 525-530.

80. Fabre, N., Urizzi, P., Souchard, J., Fréchard, A., Claparols, C., Fourasté, I. ve diğerleri. (2000) An antioxidant sinapic acid ester isolated from Iberis amara.

Fitoterapia, 71 (4), 425-428.

81. Vuorela, S., Kreander, K., Karonen, M., Nieminen, R., Hämäläinen, M., Galkin, A. ve diğerleri. (2005) Preclinical evaluation of rapeseed, raspberry, and pine bark phenolics for health related effects. *Journal of Agricultural and Food Chemistry*, 53 (15), 5922-5931.
82. Kylli, P., Nousiainen, P., Biely, P., Sipilä, J., Tenkanen, M.,Heinonen, M. (2008) Antioxidant potential of hydroxycinnamic acid glycoside esters. *Journal of Agricultural and Food Chemistry*, 56 (12), 4797-4805.
83. Jalaludeen, A.M.,Pari, L. (2011) Studies on the antioxidant and free radical-scavenging effect of sinapic acid: An in vivo and in vitro model. *Journal of Pharmaceutical Sciences and Research*, 3 (9).
84. Nordberg, J.,Arner, E.S. (2001) Reactive oxygen species, antioxidants, and the mammalian thioredoxin system. *Free Radical Biology and Medicine*, 31 (11), 1287-1312.
85. Pekkarinen, S.S., Stöckmann, H., Schwarz, K., Heinonen, I.M.,Hopia, A.I. (1999) Antioxidant activity and partitioning of phenolic acids in bulk and emulsified methyl linoleate. *Journal of Agricultural and Food Chemistry*, 47 (8), 3036-3043.
86. Thiyam, U., Kuhlmann, A., Stöckmann, H.,Schwarz, K. (2004) Prospects of rapeseed oil by-products with respect to antioxidative potential. *Comptes Rendus Chimie*, 7 (6), 611-616.
87. Bortolomeazzi, R., Sebastianutto, N., Toniolo, R.,Pizzariello, A. (2007) Comparative evaluation of the antioxidant capacity of smoke flavouring phenols by crocin bleaching inhibition, DPPH radical scavenging and oxidation potential. *Food Chemistry*, 100 (4), 1481-1489.
88. Koski, A., Pekkarinen, S., Hopia, A., Wähälä, K.,Heinonen, M. (2003) Processing of rapeseed oil: effects on sinapic acid derivative content and oxidative stability. *European Food Research and Technology*, 217 (2), 110-114.
89. Terpinc, P., Polak, T., Šegatin, N., Hanzlowsky, A., Ulrih, N.P.,Abramovič, H. (2011) Antioxidant properties of 4-vinyl derivatives of hydroxycinnamic acids.

Food Chemistry, 128 (1), 62-69.

90. Figueroa-Espinoza, M.-C.,Villeneuve, P. (2005) Phenolic acids enzymatic lipophilization. *Journal of agricultural and food chemistry*, 53 (8), 2779-2787.
91. Gaspar, A., Martins, M., Silva, P., Garrido, E.M., Garrido, J., Firuzi, O. ve diğerleri. (2010) Dietary phenolic acids and derivatives. Evaluation of the antioxidant activity of sinapic acid and its alkyl esters. *Journal of Agricultural and Food Chemistry*, 58 (21), 11273-11280.
92. Stocker, R.,Keaney, J.F. (2004) Role of oxidative modifications in atherosclerosis. *Physiological Reviews*, 84 (4), 1381-1478.
93. Niki, E. (1990) [3] Free radicals initiators as source of water-or lipid-soluble peroxyl radicals. *Methods in Enzymology*, 186, 100-108.
94. Andreasen, M.F., Landbo, A.-K., Christensen, L.P., Hansen, Å.,Meyer, A.S. (2001) Antioxidant effects of phenolic rye (Secale cereale L.) extracts, monomeric hydroxycinnamates, and ferulic acid dehydrodimers on human low-density lipoproteins. *Journal of Agricultural and Food Chemistry*, 49 (8), 4090-4096.
95. Nardini, M., D'Aquino, M., Tomassi, G., Gentili, V., Di Felice, M.,Scaccini, C. (1995) Inhibition of human low-density lipoprotein oxidation by caffeic acid and other hydroxycinnamic acid derivatives. *Free Radical Biology and Medicine*, 19 (5), 541-552.
96. Hynes, M.J.,Ó Coinceanainn, M.n. (2002) Investigation of the release of iron from ferritin by naturally occurring antioxidants. *Journal of Inorganic Biochemistry*, 90 (1), 18-21.
97. Chalas, J., Claise, C., Edeas, M., Messaoudi, C., Vergnes, L., Abella, A. ve diğerleri. (2001) Effect of ethyl esterification of phenolic acids on low-density lipoprotein oxidation. *Biomedicine and Pharmacotherapy*, 55 (1), 54-60.
98. Kitagawa, S., Sugiyama, Y.,Sakuma, T. (1996) Inhibitory effects of catechol derivatives on hydrophilic free radical initiator-induced hemolysis and their interaction with hemoglobin. *Chemical and Pharmaceutical Bulletin*, 44 (5), 881-884.

99. Yun, K.-J., Koh, D.-J., Kim, S.-H., Park, S.J., Ryu, J.H., Kim, D.-G. ve diğerleri. (2008) Anti-inflammatory effects of sinapic acid through the suppression of inducible nitric oxide synthase, cyclooxygase-2, and proinflammatory cytokines expressions via nuclear factor-κB inactivation. *Journal of Agricultural and Food Chemistry*, 56 (21), 10265-10272.

100. Kampa, M., Alexaki, V.-I., Notas, G., Nifli, A.-P., Nistikaki, A., Hatzoglou, A. ve diğerleri. (2004) Antiproliferative and apoptotic effects of selective phenolic acids on T47D human breast cancer cells: potential mechanisms of action. *Breast Cancer Res*, 6 (2), R63-74.

101. Cao, X., Tsukamoto, T., Seki, T., Tanaka, H., Morimura, S., Cao, L. ve diğerleri. (2008) 4-Vinyl-2, 6-dimethoxyphenol (canolol) suppresses oxidative stress and gastric carcinogenesis in Helicobacter pylori-infected carcinogen-treated Mongolian gerbils. *International Journal of Cancer*, 122 (7), 1445-1454.

102. Kuwahara, H., Kanazawa, A., Wakamatu, D., Morimura, S., Kida, K., Akaike, T. ve diğerleri. (2004) Antioxidative and antimutagenic activities of 4-vinyl-2, 6-dimethoxyphenol (canolol) isolated from canola oil. *Journal of Agricultural and Food Chemistry*, 52 (14), 4380-4387.

103. Yoon, B.H., Jung, J.W., Lee, J.-J., Cho, Y.-W., Jang, C.-G., Jin, C. ve diğerleri. (2007) Anxiolytic-like effects of sinapic acid in mice. *Life Sciences*, 81 (3), 234-240.

104. Maddox, C.E., Laur, L.M.,Tian, L. (2010) Antibacterial activity of phenolic compounds against the phytopathogen Xylella fastidiosa. *Current Microbiology*, 60 (1), 53-58.

105. Johnson, M., Dahiya, J., Olkowski, A.,Classen, H. (2008) The effect of dietary sinapic acid (4-hydroxy-3, 5-dimethoxy-cinnamic acid) on gastrointestinal tract microbial fermentation, nutrient utilization, and egg quality in laying hens. *Poultry Science*, 87 (5), 958-963.

106. Nowak, H., Kujawa, K., Zadernowski, R., Roczniak, B.,KozŁowska, H. (1992) Antioxidative and bactericidal properties of phenolic compounds in rapeseeds. *Lipid/Fett*, 94 (4), 149-152.

107. Lyon, G.,McGill, F. (1988) Inhibition of growth ofErwinia carotovora in vitro by phenolics. *Potato Research*, 31 (3), 461-467.

108. Barber, M.S., McConnell, V.S.,DeCaux, B.S. (2000) Antimicrobial intermediates of the general phenylpropanoid and lignin specific pathways. *Phytochemistry*, 54 (1), 53-56.

109. Tesaki, S., Tanabe, S., Ono, H., Fukushi, E., Kawabata, J.,WATANABE, M. (1998) 4-Hydroxy-3-nitrophenylacetic and sinapic acids as antibacterial compounds from mustard seeds. *Bioscience, Biotechnology, and Biochemistry*, 62 (5), 998-1000.

110. Engels, C., Schieber, A.,Gänzle, M.G. (2012) Sinapic acid derivatives in defatted Oriental mustard (Brassica juncea L.) seed meal extracts using UHPLC-DAD-ESI-MS n and identification of compounds with antibacterial activity. *European Food Research and Technology*, 234 (3), 535-542.

111. Soreq, H.,Seidman, S. (2001) Acetylcholinesterase—new roles for an old actor. *Nature Reviews Neuroscience*, 2 (4), 294-302.

112. He, L., Li, H., Guo, S., Liu, L., Qiu, J., Li, F. ve diğerleri. (2008) [Inhibitory effects of sinapine on activity of acetylcholinesterase in cerebral homogenate and blood serum of rats]. *Zhongguo Zhong yao za zhi= Zhongguo zhongyao zazhi= China Journal of Chinese Materia Medica*, 33 (7), 813-815.

113. Ferreres, F., Fernandes, F., Sousa, C., Valentao, P., Pereira, J.A.,Andrade, P.B. (2009) Metabolic and bioactivity insights into Brassica oleracea var. acephala. *Journal of Agricultural and Food Chemistry*, 57 (19), 8884-8892.

114. Lee, H.E., Kim, D.H., Park, S.J., Kim, J.M., Lee, Y.W., Jung, J.M. ve diğerleri. (2012) Neuroprotective effect of sinapic acid in a mouse model of amyloid β1–42 protein-induced Alzheimer's disease. *Pharmacology Biochemistry and Behavior*, 103 (2), 260-266.

115. Kim, D.H., Yoon, B.H., Jung, W.Y., Kim, J.M., Park, S.J., Park, D.H. ve diğerleri. (2010) Sinapic acid attenuates kainic acid-induced hippocampal neuronal damage in mice. *Neuropharmacology*, 59 (1), 20-30.

116. Karakida, F., Ikeya, Y., Tsunakawa, M., Yamaguchi, T., Ikarashi, Y., Takeda, S.

ve diğerleri. (2007) Cerebral protective and cognition-improving effects of sinapic acid in rodents. *Biological and Pharmaceutical Bulletin*, 30 (3), 514.

117. Kim, Y.-O., Lee, S.-W., Oh, M.-S.,Lee, H.J. (2011) Effects of Sinapic Acid of 4 Vessel Occlusion Model-Induced Ischemia and Cognitive Impairments in the Rat. *Clinical Psychopharmacology and Neuroscience*, 9 (2), 86-90.
118. Roy, S.J.,Stanely Mainzen Prince, P. (2012) Protective effects of sinapic acid on lysosomal dysfunction in isoproterenol induced myocardial infarcted rats. *Food and Chemical Toxicology*, 50 (11), 3984-3989.
119. Shin, D.-S., Kim, K.W., Chung, H.Y., Yoon, S.,Moon, J.-O. (2013) Effect of sinapic acid against carbon tetrachloride-induced acute hepatic injury in rats. *Archives of Pharmacal Research*, 36 (5), 626-633.
120. Wilson, J.S., Ganesan, K.,Palanisamy, M. (2011) Effect of sinapic acid on biochemical markers and histopathological studies in normal and streptozotocin induced diabetes in wistar rats. *International Journal of Pharmacy and Pharmaceutical Sciences*, 3.
121. Pari, L.,Mohamed Jalaludeen, A. (2011) Protective role of sinapic acid against arsenic–Induced toxicity in rats. *Chemico-biological interactions*, 194 (1), 40-47.
122. Kanchana, G., Jerine Shyni, W., Rajadurai, M.,Periasamy, R. (2011) Evaluation of antihyperglycemic effect of sinapic Acid in normal and streptozotocin-induced diabetes in albino rats. *Global J Pharmacol*, 5, 33-39.
123. Fan, G.J., Jin, X.L., Qian, Y.P., Wang, Q., Yang, R.T., Dai, F. ve diğerleri. (2009) Hydroxycinnamic Acids as DNA-Cleaving Agents in the Presence of CuII Ions: Mechanism, Structure–Activity Relationship, and Biological Implications. *Chemistry-A European Journal*, 15 (46), 12889-12899.
124. Zheng, L.-F., Dai, F., Zhou, B., Yang, L.,Liu, Z.-L. (2008) Prooxidant activity of hydroxycinnamic acids on DNA damage in the presence of Cu (II) ions: mechanism and structure–activity relationship. *Food and Chemical Toxicology*, 46 (1), 149-156.
125. Zhang, J., Melton, L.D., Adaim, A.,Skinner, M.A. (2008) Cytoprotective effects of polyphenolics on H2O2-induced cell death in SH-SY5Y cells in relation to

their antioxidant activities. *European Food Research and Technology*, 228 (1), 123-131.

126. Lee-Manion, A.M., Price, R.K., Strain, J., Dimberg, L.H., Sunnerheim, K.,Welch, R.W. (2009) In vitro antioxidant activity and antigenotoxic effects of avenanthramides and related compounds. *Journal of Agricultural and Food Chemistry*, 57 (22), 10619-10624.
127. Repetto, G., del Peso, A.,Zurita, J.L. (2008) Neutral red uptake assay for the estimation of cell viability/cytotoxicity. *Nature Protocols*, 3 (7), 1125-1131.
128. Arranz, M.,Festing, M. (1990) Prior use of the neutral red assay and reduction of total protein determination in 96-well plate assays. *Toxicology in Vitro*, 4 (3), 211-212.
129. Mosmann, T. (1983) Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *Journal of Immunological Methods*, 65 (1), 55-63.
130. Liu, Y., Peterson, D.A., Kimura, H.,Schubert, D. (1997) Mechanism of cellular 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) reduction. *Journal of Neurochemistry*, 69 (2), 581-593.
131. Denizot, F.,Lang, R. (1986) Rapid colorimetric assay for cell growth and survival: modifications to the tetrazolium dye procedure giving improved sensitivity and reliability. *Journal of Immunological Methods*, 89 (2), 271-277.
132. Sieuwerts, A.M., Klijn, J.G., Peters, H.A.,Foekens, J.A. (1995) The MTT Tetrazolium Salt Assay Scrutinized: How to Use this Assay Reliably to Measure Metabolie Activity of Cell Cultures in vitro for the Assessment of Growth Characteristics, IC50-Values and Cell Survival. *Clinical Chemistry and Laboratory Medicine*, 33 (11), 813-824.
133. Kirkpatrick, D.L., Duke, M.,Suan Goh, T. (1990) Chemosensitivity testing of fresh human leukemia cells using both a dye exclusion assay and a tetrazolium dye (MTT) assay. *Leukemia Research*, 14 (5), 459-466.
134. Ferrari, M., Fornasiero, M.C.,Isetta, A.M. (1990) MTT colorimetric assay for testing macrophage cytotoxic activity in vitro. *Journal of Immunological*

Methods, 131 (2), 165-172.

135. Faust, F., Kassie, F., Knasmüller, S., Boedecker, R.H., Mann, M.,Mersch-Sundermann, V. (2004) The use of the alkaline comet assay with lymphocytes in human biomonitoring studies. *Mutation Research/Reviews in Mutation Research*, 566 (3), 209-229.
136. Azqueta, A., Gutzkow, K.B., Priestley, C.C., Meier, S., Walker, J.S., Brunborg, G. ve diğerleri. (2013) A comparative performance test of standard, medium-and high-throughput comet assays. *Toxicology in Vitro*, 27 (2), 768-773.
137. Uhl, M., Helma, C.,Knasmüller, S. (1999) Single-cell gel electrophoresis assays with human-derived hepatoma (Hep G2) cells. *Mutation Research/Genetic Toxicology and Environmental Mutagenesis*, 441 (2), 215-224.
138. Fenech, M. (2000) The in vitro micronucleus technique. *Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis*, 455 (1), 81-95.
139. Fenech, M. (2007) Cytokinesis-block micronucleus cytome assay. *Nature Protocols*, 2 (5), 1084-1104.
140. Virgilio, A.L.D., Iwami, K., Wätjen, W., Kahl, R.,Degen, G.H. (2004) Genotoxicity of the isoflavones genistein, daidzein and equol in V79 cells. *Toxicology Letters*, 151 (1), 151-162.
141. Saquib, Q., Al-Khedhairy, A.A., Siddiqui, M.A., Abou-Tarboush, F.M., Azam, A.,Musarrat, J. (2012) Titanium dioxide nanoparticles induced cytotoxicity, oxidative stress and DNA damage in human amnion epithelial (WISH) cells. *Toxicology in Vitro*, 26 (2), 351-361.
142. Collins, A.R. (2014) Measuring oxidative damage to DNA and its repair with the comet assay. *Biochimica et Biophysica Acta (BBA)-General Subjects*, 1840 (2), 794-800.
143. Albertini, R.J., Anderson, D., Douglas, G.R., Hagmar, L., Hemminki, K., Merlo, F. ve diğerleri. (2000) IPCS guidelines for the monitoring of genotoxic effects of carcinogens in humans. *Mutation Research/Reviews in Mutation Research*, 463 (2), 111-172.

144. Daroui, P., Desai, S.D., Li, T.-K., Liu, A.A.,Liu, L.F. (2004) Hydrogen peroxide induces topoisomerase I-mediated DNA damage and cell death. *Journal of Biological Chemistry*, 279 (15), 14587-14594.

145. Maistro, E., Angeli, J., Andrade, S.,Mantovani, M. (2011) In vitro genotoxicity assessment of caffeic, cinnamic and ferulic acids. *Genetics and Molecular Research*, 10 (2), 1130-1140.

146. do Céu Silva, M., Gaspar, J., Silva, I.D., Leão, D.,Rueff, J. (2003) Induction of chromosomal aberrations by phenolic compounds: possible role of reactive oxygen species. *Mutation Research/Genetic Toxicology and Environmental Mutagenesis*, 540 (1), 29-42.