STUDY OF THE NATURAL ALKENYLBENZENES COMPOUNDS: MECHANISMS OF DNA LESIONS AND IMPLICATIONS FOR HUMAN HEALTH

CÉLIA MARIA DA SILVA MARTINS

Tese de obtenção do grau de Doutor em Ciências da Vida

na Especialidade em Genética, Oncologia e Toxicologia Humana

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Célia Maria da Silva Martins

Orientador: António Sebastião Rodrigues, Professor Auxiliar

Co-orientador: José Rueff, Professor Catedrático

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"Só com o acto de pintar vemos o que nunca tínhamos visto; só com o acto de investigar percebemos o que nunca tínhamos percebido" Luís Damas Mora, em Cadernos de um cirurgião, da autoria do Professor Francisco d'Oliveira Martins.

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ABSTRACT

Alkenylbenzenes are a family of natural compounds found in spices, such as nutmeg, clove and anise, and essential oils of various plants and encompass more than 30 compounds with different structural features. The natural sources containing these compounds have been used not only in foods but in medicines for thousands of years, in essential oils, infusions, teas or even in pharmaceutical formulations. Consequently, this family of natural compounds is present and widely used not only in Western diets but in medicinal preparations, providing a significant potential risk, as some of these compounds have genotoxic properties.

In this thesis our goal was to assess the mechanisms of genotoxicity of several alkenylbenzenes, including eugenol, estragole and myristicin, all widely present in spices. We showed that eugenol, present in clove, induced chromosomal aberrations in V79 Chinese hamster cells and H2AX phosphorylation in AA8 Chinese hamster ovary cells, indicating the formation of double-strand breaks. More interestingly eugenol induced endoreduplication, which suggested that this compound is a topoisomerase inhibitor. Another alkenylbenzene studied was estragole, present in tarragon, basil and anise. This compound was shown to produce DNA strand breakage, sister chromatid exchanges and DNA adducts, which could be responsible for its genotoxicity. Our results also indicated that myristicin, an alkenylbenzene present in basil, anise, cinnamon, clove, fennel, nutmeg, parsley and star anise, is not genotoxic but is apoptotic, and downregulates DNA damage response genes, with significant under-expression of genes associated with nucleotide excision repair, double strand break repair and DNA damage signalling and stress response. Finally, as a proof of concept, we addressed DNA methylation alterations in several genes induced by prolonged exposure to a low dose (10 μM) of the alkenylbenzenes eugenol and elemicin in human breast cancer MCF-7 cells. We showed that eugenol and elemicin can increase expression of RASSF1A in MCF-7 breast cancer cells, as measured by RT-PCR, and can induce DNA demethylation, although without a direct clear cut result. Unexpectedly, we also observed an increased in phospho-ATM and phospho-H2AX after prolonged exposure to eugenol and elemicin, indicating possible genotoxic effects at low doses.

Globally, our results may contribute to a further understanding of the potential risk of increasing our consumption of these alkenylbenzenes present in the diet, as was recommended by the Joint FAO/WHO Expert Committee on Food Additives (JECFA).

Keywords: Alkenylbenzenes, eugenol, myristicin, estragole, elemicin, genotoxicity, apoptosis, DNA methylation.

RESUMO

Os alquenilbenzenos são compostos naturais encontrados em especiarias como a noz-moscada, cravinho e anis e nos seus óleos essenciais englobando mais de 30 compostos com diferentes características estruturais. Os produtos naturais contendo estes compostos têm sido utilizados ao longo dos anos não apenas em alimentos mas também em medicinas, como óleos essenciais, infusões, chás ou mesmo em formulações farmacêuticas. Por conseguinte, esta família de compostos não só está presente como é amplamente utilizada tanto na alimentação ocidental como em preparações medicinais, apresentando um potencial de risco significativo uma vez que alguns destes compostos são genotóxicos.

O objetivo desta tese foi avaliar os mecanismos de genotoxicidade de vários alquenilbenzenos, incluindo o eugenol, o estragole e a miristicina, todos eles presentes em especiarias. Demonstrámos que o eugenol, presente no cravinho, induz aberrações cromossómicas em células V79 e leva à fosforilação da histona H2AX em células de ovário de Hamster Chinês AA8, indicando a formação de quebras de dupla cadeia no DNA. O eugenol também induziu endoreduplicação, o que sugere que este composto é um inibidor da topoisomerase. Outro alquenilbenzeno estudado foi o estragole, presente no estragão, manjericão e anis. Este composto induziu quebras no DNA, trocas de cromátides irmãs e aductos no DNA, que provavelmente são responsáveis pela sua ação genotóxica. Os nossos resultados também indicaram que a miristicina, presente no manjericão, anis, canela, cravinho, funcho, noz-moscada, salsa e anis, não é genotóxica mas é apoptótica, e levou a uma subexpressão de genes envolvidos nas vias de reparação de DNA, em particular genes envolvidos na reparação por excisão de nucleotídeos, reparação de quebras de cadeia dupla de DNA e na sinalização de lesões no DNA e stress celular. Por último, como prova de conceito, avaliámos alterações na metilação do DNA de vários genes após exposição prolongada a uma dose baixa (10 μM) dos alquenilbenzenos eugenol e elemicina em células humanas de cancro da mama, MCF-7. Observámos que o eugenol e a elemicina aumentam a expressão do gene RASSF1A e alteram o estado de metilação do DNA, embora sem se conseguir ter uma relação directa entre estes dois fenómenos. Inesperadamente, observámos um aumento do ATM e do H2AX fosforilado após

exposição prolongada ao eugenol e à elemicina, indicando um risco aumentado para possíveis efeitos genotóxicos a doses baixas.

Globalmente, os nossos resultados podem contribuir para uma maior compreensão do risco associado ao consumo destes alquenilbenzenos presentes na alimentação, como foi preconizado pela JECFA.

Palavras-chave: Alquenilbenzenos, eugenol, miristicina, estragole, elemicina, genotoxicidade, apoptose, metilação do DNA.

LIST OF PUBLICATIONS AND COMMUNICATIONS

From the results published in this thesis, the following full papers were published in international refereed journals:

- Maralhas A., Monteiro A., **Martins C.**, Kranendonk M., Laires A., Rueff J. and Rodrigues A.S. (**2006**) Genotoxicity and endoreduplication inducing activity of the food flavouring eugenol. *Mutagenesis*, 21(3):199-204. http://www.ncbi.nlm.nih.gov/pubmed/16595588. DOI: 10.1093/mutage/ge1017.
- **Martins C.**, Doran C., Laires A., Rueff J. and Rodrigues A.S. (**2011**) Genotoxic and apoptotic activities of the food flavourings myristicin and eugenol in AA8 and XRCC1 deficient EM9 cells. *Food and Chemical Toxicology,* 49(2):385-92. http://www.ncbi.nlm.nih.gov/pubmed/21087650. DOI: 10.1016/j.fct.2010.11.013.
- **Martins C.**, Cação R., Cole K.J., Phillips D.H., Laires A., Rueff J. and Rodrigues A.S. Estragole: A weak direct-acting food-borne genotoxin and potential carcinogen (**2012**) Mutation Research - Genetic Toxicology and Environmental Mutagenesis, 747(1):86-92. DOI:10.1016/j.mrgentox.2012.04.009.
- **Martins C.**, Doran C., Silva I.C., Miranda C., Rueff J. and Rodrigues A.S. (**2014**) Myristicin from nutmeg induces apoptosis via the mitochondrial pathway and down regulates genes of the DNA damage response pathways in human leukaemia K562 cells. Chem Biol Interact. 218 :1-9. DOI: 10.1016/j.cbi.2014.04.014.

From the results published in this thesis, the following abstracts were published in international refereed journals:

- **Martins C.**, Armada A., Viveiros M. Rueff J. and Rodrigues A. (**2013**) Allylbenzenes as potential chemosensitizers and P-glycoprotein inhibitors. 17th ECCO / 38th ESMO / 32nd ESTRO European Cancer Congress on Reinforcing Multidisciplinarity, Sep 27- Oct 01, Amsterdam, Netherlands. EUROPEAN JOURNAL OF CANCER, Vol.: 49 Supplement: 2 Pages: S178-S178: Abstract.
- **Martins C.**, Silva I.C., Rueff J. and Rodrigues A.S. (**2014**) Food flavouring myristicin down-regulates genes of the DNA damage response pathways in human leukaemia K562 cells. 50th

Congress of the European Societies of Toxicology (EUROTOX), **7th-10th September**, Edinburgh International Conference Center, Edinburgh. TOXICOLOGY LETTERS, Vol.: 229S Pages: S179-S179: Abstract nr. P-3.99.

From the results in this thesis, the following oral communications were presented in the scientific meetings:

- **Martins C.**, Rodrigues A.S., Laires A. and Rueff J. (**2004**) Direct genotoxicity of the food mutagen estragole. XXXI Jornadas Portuguesas de Genética, ITQB – Oeiras, Portugal.
- **Martins C.** (2015) Epigenetic processes as molecular targets of natural compounds. 1º Workshop de Genética, Universidade Nova de Lisboa, NOVAsaúde.

The results obtained in this thesis were also presented in scientific meetings in the following poster communications:

- Rodrigues A.S., Monteiro A.F., Maralhas A., **Martins C.**, Laires A. and Rueff J. Genotoxicity and endoreduplication inducing activity of the food flavouring eugenol. 35th Annual Meeting of the European Environmental Mutagen Society: Environment and human genetic disease - causes, mechanisms and effects (**2005**), Kos Island, Greece.
- **Martins C.**, Monteiro A.F., Maralhas A., Saraiva P., Vaz S., Laires A., Rueff J. and Rodrigues A.S. Cytotoxicity and genotoxicity of the food flavourings eugenol and estragole in V79 and CHO cell lines. 36th Annual Meeting of the European Environmental Mutagen Society: From Genes to molecular epidemiology (**2006**), Prague, Czech Republic.
- **Martins C.**, Doran C., Laires A., Rueff J. and Rodrigues A. Comparative studies of the three alkenylbenzenes Estragole, Eugenol and Myristicin in terms of DNA repair and apoptosis. 38th Annual Meeting of the European Environmental Mutagen Society: Environmental mutagens and human health (**2008**), Croácia, Cavtat.
- Rodrigues A., **Martins C.**, Cação R., Laires A. and Rueff J. Genotoxicity and apoptosis induced by the food flavoutring estragole in V79 and the CHO cell lines AA8 and EM9 cells. Gordon Research Conference on DNA Damage, Mutation & Cancer (**2008**), Ventura, CA, USA.
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K562 leukaemia cells: Effect of the alkenylbenzene myristicin. 10th International Conference on Environmental Mutagens (ICEM); 39th Annual Meeting of the European Environmental Mutagen Society (EEMS); 18th Annual Meeting of the Italian Environmental Mutagen Society (SIMA): The Renaissance of Environmental Mutagenesis (**2009**), Italy, Florence.

- **Martins C.**, Doran C., Miranda C., Laires A., Rueff J. and Rodrigues A.S. Study of the allylbenzene food flavouring myristicin: impact on gene expression and cell survival. 15ª Reunião Anual da Sociedade Portuguesa de Genética Humana (SPGH) (**2011**), Lisboa, Portugal.
- **Martins C.**, Doran C., Miranda C., Laires A., Rueff J. and Rodrigues A.S. The food flavouring myristicin from nutmeg induces apoptosis and modulates gene expression in leukemia K562 cells. 37ª Jornadas Portuguesas de Genética (**2012**), Lisboa, Portugal.
- **Martins C.**, Armada A., Viveiros M., Rueff J. and Rodrigues A.S. Allylbenzenes as potential chemosensitizers and p-glycoprotein inhibitors. 17th ECCO / 38th ESMO / 32nd ESTRO European Cancer Congress on Reinforcing Multidisciplinarity, Sep 27- Oct 01 (**2013**), Amsterdam, Netherlands.
- **Martins C.**, Silva I.C., Rueff J. and Rodrigues A.S. Food flavouring myristicin down-regulates genes of the DNA damage response pathways in human leukaemia K562 cells. 50th Congress of the European Societies of Toxicology (EUROTOX), 7th-10th September (**2014**), Edinburgh International Conference Center, Edinburgh.
- Silva I., **Martins C.**, Rueff J. and Rodrigues A.S. Modulation of DNA methylation by dietary phytochemicals. 16ª Reunião Anual da Sociedade Portuguesa de Genética Humana (SPGH) (**2014**), Lisboa, Portugal.
- **Martins C.**, Silva I.C., Rueff J. and Rodrigues A.S. Epigenetic changes after prolonged exposure to alkenylbenzenes – an important signature of potential toxicological effects. 51th Congress of the European Societies of Toxicology (EUROTOX), 13-16 September (**2015**), Porto, Portugal.

<u>This thesis also contains data and methods published in the following book chapters:</u>

- Rodrigues A.S., Gomes B.C., **Martins C.**, Gromicho M., Oliveira N.G., Guerreiro P.S. and Rueff J. (**2013**) DNA Repair and Resistance to Cancer Therapy. New Research Directions in DNA Repair. Prof. Clark Chen (Ed.), ISBN: 978-953-51-1114-6, InTech. DOI: 10.5772/53952.

Available from: http://www.intechopen.com/books/new-research-directions-in-dna-repair/dna-repair-and-resistance-to-cancer-therapy.

- Armada A., **Martins C.**, Spengler G., Molnar J., Amaral L., Rodrigues A.S. and Viveiros M. (**2016**) Fluorometric methods for analysis of permeability, drug transport kinetics and inhibition of the ABCB1 membrane transporter. In: José Rueff and António S. Rodrigues, eds. Cancer Drug Resistance - Methods in Molecular Biology, Chapter 7, volume 1395, pp 87-103, Springer.

This thesis also contains data and methods published in the following papers:

- Oliveira N.G., Castro M., Rodrigues A.S., Gonçalves I.C., **Martins C.**, Toscano Rico J.M. and Rueff J. (**2005**) Effect of poly(ADP-ribosyl)ation inhibitors on the genotoxic effects of the boron neutron capture reaction. *Mutat Res – Genetic Toxicology and Environmental Mutagenesis,* 583 (1): 36-48. DOI:10.1016/j.mrgentox.2005.01.015.
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%	percentage
µg	microgram
µmol	micromole
µM	micromolar
ADI	acceptable daily intake
AP	apurinic/apyrimidinic site
ATM	ataxia telangiectasia mutated
B[a]P	benzo[a]pyrene
BC	before Christ
BER	base excision repair
BLM	Bloom syndrome RecQ like helicase
BMDL10	bechmark dose model 10
BrdU	5-bromo-2'-deoxyuridine
bw	body weight
CAM	complementary and alternative medicine
CAs	chromosomal aberrations
CE	Common Era
CEFS	committee of experts on flavouring substances
CNS	central nervous system
COE	council of Europe
CP	cyclophosphamide
CYP	cytochrome P450
DDR	DNA Damage Response
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
DSB	double strand breaks
EFSA	European food safety authority
EH	epoxide hydrolase

EMEA	European committee on herbal medicinal products
EPA	environmental protection agency
EU	European Union
FA	Fanconi anaemia
FAO	food and agriculture organization
FDA	food and drug administration
FEMA	flavor and extract manufacturers association
FPG	formamidopyrimidine-DNA-glycosylase
Gua	guanine
GRAS	generally regarded as safe
GSH	glutathione
GST	glutathione S-transferases
h	hour
H2AX	histone H2A
HepG2	human hepatoma cells
HPLC	high performance liquid chromatography
HR	homologous recombination
IARC	international agency for research on cancer
ICRF-193	meso-4,4′-(3,2-Butanediyl)-bis(2,6-piperazinedione)
i.p.	intraperitoneal injection
JECFA	joint FAO/WHO expert committee on food additives
KCl	potassium chloride
Kg	kilogram
LC-MS/MS	liquid chromatography-tandem mass spectrometry
Max	maximum
mg	miligram
MGMT	O^6-alkylguanine DNA alkyltransferase
min	minute
ml	mililiter

MMC	mitomycin C
mmol	milimole
MMR	mismatch repair
MN	micronuclei
MOE	margin of exposure
MRP2	multidrug resistance-associated protein 2
MS	mass spectrometry
MSP1	merozoite surface protein-1
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide
NER	nucleotide excision repair
ng	nanogram
NHEJ	non-homologous end joining
nM	nanomolar
nmol	nanomole
NTP	national toxicology program
O	oxygen
$^{\cdot}O_2$	hyperoxide
$^{\cdot}OH$	hydroxyl radical
^{32}P	phosphorus-32
PAPS	3´-phosphoadenosine 5´-phosphosulfate
PBK	physiologically-based kinetic
PBS	phosphate buffered saline
Pgps	P-glycoprotein
pmol	picomole
ppm	parts per million
RASSF1	Ras association domain-containing protein 1
RNA	ribonucleic acid
ROS	reactive oxygen species
RT-PCR	reverse transcription polymerase chain reaction

S9	microsomal fraction
s.c.	subcutaneous administration
SCE	sister chromatid exchanges
SCF	scientific committee on food
SHE	Syrian hamster embryo cells
SSB	single strand break
SULTS	sulfotransferases
T1/2	half-life
Topo II	topoisomerase II
UDS	unschedule DNA synthesis
UGT	uridine 5'-diphospho-glucuronosyltransferase
USA	United States of America
UV	ultraviolet
v/cm	volume/centimeter
v/v	volume/volume
w/v	weight/volume
WHO	world health organization

1. Chapter

INTRODUCTION

BRIEF OVERVIEW

The Science of Toxicology

The first approaches to the science of toxicology had to do with categorization of plants and minerals in relation to their beneficial or harmful properties. Currently, toxicology is the science of determining adverse effects caused by agents that we, the environmental and animals, are exposed to, and the methodology that can predict adverse effects, and thus establishment of future approaches to minimize exposure. Therefore, toxicology can be seen has a preventive science.

The concept of toxic effects probably exists since the appearance of modern Man. Known records of toxic effects date back to 1500 BC. The ancient Egyptian *Ebers Papyrus*, dating from around 1550 BC, contains about 800 complex prescriptions and more than 700 natural agents such as *Aloe vera* cactus (Ji et al., 2009). In ancient Greece, the physician Hippocrates of Cos (*circa* 460–377 BC) collected more than 400 natural agents and described their use, categorizing them as harmful or safe (Casarett et al., 2001b; Ji et al., 2009). In the orient Shen Nong Ben Cao Ling, considered one of the fathers of Chinese Medicine, categorized in a monograph (*Shen Nong Materia Medica*) more than 300 herbs as medicines (Shou-zhong, 2008).

The importance of such "old" records was proven by Youyou Tu, the Nobel Prize winner in Physiology or Medicine in 2015. Youyou Tu discovered a novel therapy against Malaria, an *Artemisia annua* L. extract, which had been already described in ancient Chinese manuscripts. In a hundreds-of-years-old text, "The Manual of Clinical Practice and Emergency Remedies" by Ge Hong of the East Jin Dynasty (284–346 CE), she and her team found a mention of a sweet wormwood (*Artemisia annua*) that was used to treat malaria. Artemisinin-based antimalarial have led to the survival and improved health of millions of people (http://www.nobelprize.org/nobel_prizes/medicine/laureates/2015/tu-facts.html).

"All substances are poisons; there is none which is not a poison. The right dose differentiates a poison from a remedy".

Paracelsus

Paracelsus (Philippus Aureolus Theophrastus Bombastus von Hohenheim-Paracelsus (1493–1541), is considered one of the most prominent figures in the history of science and medicine in the late Middle Ages. He was a physician-alchemist and the son of a physician and formulated revolutionary views that remain an integral part of the structure of toxicology, pharmacology, and therapeutics (Casarett et al., 2001b). The view initiated by Paracelsus was held as corollaries: (1) Experimentation is essential in the examination of responses to chemicals, (2) one should make a distinction between the therapeutic and toxic properties of chemicals, (3) these properties are sometimes but not always indistinguishable except by dose, and (4) one can ascertain a degree of specificity of chemicals and their therapeutic or toxic effects (Casarett et al., 2001a).

After Paracelsus, throughout Europe, in the Middle Ages and early Renaissance, poisons became almost common. Many uses were applied for poisons; they were used to claim political power, for women to kill their unwanted husbands or for suicidal motives (Casarett et al., 2001a).

Genetic Toxicology and DNA Damage

The evolution of toxicology throughout the years was natural and there are many examples provided by the literature. Modern toxicology gave us many disciplines and one of them is Genetic Toxicology. One can say that genetic toxicology is the field of toxicology that assesses the effects of chemical and physical agents on the hereditary material (DNA) and on the genetic processes of living cells (Casarett et al., 2001c; Kramer, 1998).

Genetic toxicology began as a basic research field with the study of the effect of ionizing radiations and chemicals in plant, insect, and mammalian cells resulting in mutations and chromosome alterations. The development of short-term assays for genetic toxicology served to identify mutagens and to take another step forward in identifying the relationship between mutagens and cancer-causing agents, or carcinogens (Casarett et al., 2001c). The mechanisms underlying genetic toxicology assays help us understand the mechanisms of mutagenesis, and to develop new methods for the assessment of genetic alterations, being a valuable weapon for cancer and genetic risk assessment (Casarett et al., 2001c; Kramer, 1998).

Two different concepts, but often linked, born from genetic toxicology are: mutagenicity and genotoxicity. Mutagenicity tests measure alterations in DNA, called mutations, those alterations can be transmissible from cell to cell or generation to generation. Genotoxicity tests measure a large spectrum of endpoints such as DNA breaks, adducts, epigenetic alterations, chromosome rearrangements, among others, that eventually will lead to cell dysfunction, not being necessarily a permanent lesion. Overall, a mutagen is a genotoxic compound, but a genotoxic compound may or may not be a mutagen.

DNA integrity and stability are essential to life but DNA is not an inert chemical entity, it contains many potentially reactive sites and its structure can be modified in a number of ways (Clancy, 2008; Venitt and Parry, 1984).

DNA can be subject to damage from numerous sources. The main sources can be divided in endogenous sources (physiological processes) such as errors during replication (DNA mismatches), DNA strand breaks caused by oxidative damage from byproducts of metabolism, such as free reactive oxygen species (ROS) or exogenous sources such as radiation (e.g. Ultraviolet light (UV), ionizing radiation), diet, pollutant chemical agents and drugs as summarized in Figure 1.1 (Alberts et al., 2002; Jackson and Bartek, 2009; Rodrigues et al., 2013). Endogenous agents are responsible for several hundred DNA lesions per cell per day. The majority of these lesions are altered DNA bases (e.g. 8-oxoguanine and thymine glycol) and apurinic/apyrimidinic (AP) sites. The cellular processes that can lead to a DNA lesion come from oxygen consumption that results in the formation of reactive active oxygen species (e.g., superoxide $^{\cdot}0_2$, hydroxyl free radicals $^{\cdot}$OH, and hydrogen peroxide) and deamination of cytosines and S-methylcytosines leading to uracils and thymines, respectively. The process of DNA replication, although an extremely accurate mechanism, can be itself error-prone, and an incorrect base can be added by polymerases. Exogenous agents can be any electrophilic chemical that react with DNA. An electrophilic agent can form, for example, covalent addition products named adducts. Adducts such as in some alkylated bases can mispair, causing mutations when DNA is replicated. Alkylated bases can also lead to secondary alterations in DNA. For example, the alkyl group of an N7- alkylguanine adduct, which is a major adduct formed by many alkylating agents, labilizes the bond that connects the base to deoxyribose, thereby stimulating base loss.

Base loss from DNA leaves an AP site. The insertion of incorrect bases into AP sites causes mutations (Giglia-Mari et al., 2011; Laval et al., 1990).

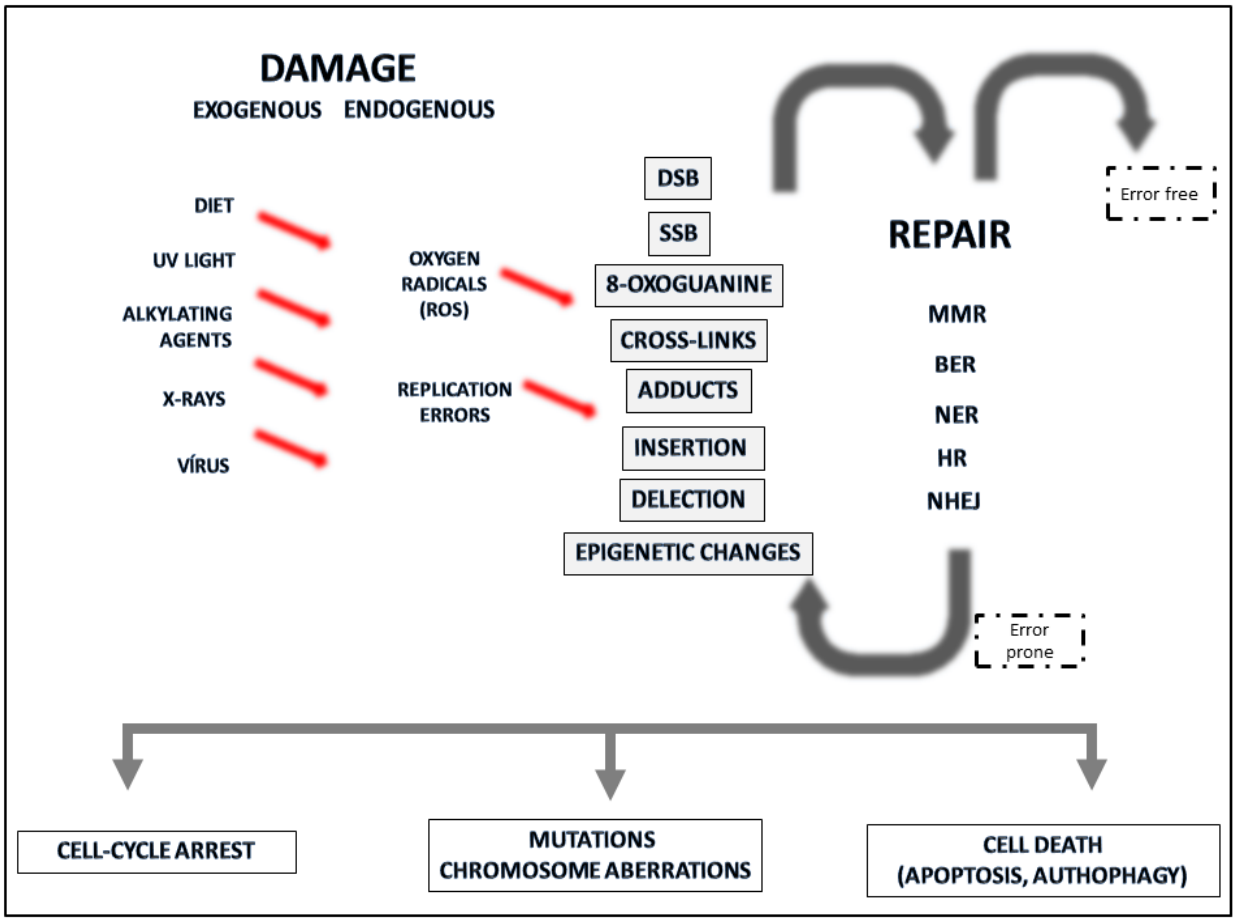

Figure 1.1 DNA damage agents, consequences and response mechanisms.

Thus, the first indication that a chemical can interact with the DNA lies in its chemical structure. Potential electrophilic sites in a molecule serve as an alert for possible mutagenicity/genotoxicity and carcinogenicity, because such sites confer reactivity with nucleophilic sites in DNA (Tennant and Ashby, 1991). But not all possible mutagens have structural alerts, and mutagenesis can be induced indirectly by mechanisms such as the generation of radicals that cause oxidative DNA damage, as already mentioned (Alberts et al., 2002). We also have to take in to account that many compounds that are not themselves mutagenic or carcinogenic can be activated into mutagens and carcinogens by mammalian metabolism. Such compounds are called promutagens

and procarcinogens. Because microorganisms and mammalian cell cultures lack many of the metabolic capabilities, an external metabolic activation system can be used in order to detect promutagens. The most common *in vitro* metabolic activation system used in bacterial or cell culture assays is derived from a mammalian post mitochondrial supernatant from rat liver homogenate, called S9 mix (Kirkland et al., 1989; Maron and Ames, 1983). Nevertheless, despite their usefulness, the *in vitro* metabolic activation systems, may not mimic *in vivo* mammalian metabolism perfectly.

Considering that each cell in the human body can undergo approximately 10^4–10^5 DNA lesions per day, those injuries have to be immediately corrected by a set of processes (Giglia-Mari et al., 2011; Jackson and Bartek, 2009) that are collectively called DNA damage response (DDR) mechanisms. DDR is a response network involving DNA repair mechanisms, DNA tolerance mechanisms and cell cycle check points that is also linked with cell death pathways (Giglia-Mari et al., 2011; Rodrigues et al., 2013). Because of the DDR, most of such spontaneous changes in DNA are temporary (Jackson and Bartek, 2009) and of the thousands of random changes in the DNA, only a few accumulate as mutations in the DNA sequence. Less than one in 1000 accidental base changes results in a permanent mutation; the rest are eliminated with remarkable efficiency by DNA repair (Alberts et al., 2002).

Nevertheless, the cell may not repair the damage and restore its DNA correctly (error free). The cell may repair but in a way that can lead to mutations (error prone) or if the damage is extensive or blocks any repair possibility, the cell may be induced to die by apoptosis (programmed cell death) (Venitt and Parry, 1984). Therefore, the repair process can be *per si* a leading cause of mutation which can lead to a dysfunctional cell (Jackson and Bartek, 2009).

In summary, all of these lesions induce cellular responses that cover a multitude of pathways, including many DNA repair pathways that are lesion specific as reviewed in Table 1.1

Although we can correlate type of lesion and mechanism of repair sometimes this is not so linear, as for agents that induce single strand DNA breaks (SSB). When two SSB arise in close proximity, or when the DNA-replication apparatus encounters a SSB or other lesions, double-strand breaks (DSB) can be formed. While DSB do not occur as frequently as other lesions, they are difficult to repair and extremely toxic (Jackson and Bartek, 2009; Rodrigues et al., 2013).

Table 1.1 Summary of possible lesions induced by various sources, and DNA mechanism involved.

Agent	Type of lesion	DNA repair mechanism	References
UV Light (e.g. Sunlight)	Pyrimidine dimers	NER Nucleotide excision repair (NER) - removal of an oligonucleotide of approximately 30 bp containing the damaged bases.	*Giglia-Mari et al., 2011*
Cigarette smoke and Diet	Bulky adducts		*Rodrigues et al. 2013* *Giglia-Mari et al., 2011*
Crosslinking agents such as chemotherapeutics (e.g. cisplatin and MMC)	Intrastrand crosslinks (covalent links between bases of the same DNA strand)		*Giglia-Mari et al., 2011*
ROS (e.g. formed by side-product of respiration or metabolism or formed by ionizing radiation or diet)	8-oxo-dG (small chemical alterations of DNA bases)	BER Base excision repair (BER) - excision of the damaged base	*Giglia-Mari et al., 2011*
	SSB	Ligation (single strand break repair) or BER	*Rodrigues et al. 2013* *Giglia-Mari et al., 2011*
	DSB	HR or NHEJ Homologous recombination (HR) - precisely restores the genomic sequence of the broken DNA ends by utilizing sister chromatids as template for repair. Non-homologous end joining (NHEJ) - promotes the potentially inaccurate religation of DSBs.	*Rodrigues et al. 2013* *Giglia-Mari et al., 2011*
Replication errors	Mispaired DNA bases	MMR Mismatch repair (MMR) - replacement with correct bases.	*Giglia-Mari et al., 2011*
Replication slippage	Small insertion/delection		*Giglia-Mari et al., 2011*
Alkylating agents (e.g. methyl-methane sulfonate and temozolomide)	Alkyl adducts	Direct repair by O^6-alkylguanine DNA alkyltransferase (MGMT)	*Rodrigues et al. 2013*
Chemotherapeutics	Interstrand crosslinks (covalent links between different DNA strands).	Interstrand crosslink repair involving the Fanconi Anaemia (FA) protein complex	*Giglia-Mari et al., 2011* *Jackson and Bartek, 2009*

Genetic Toxicology tests

More than 200 assays have been developed for an accurate analysis of possible mutagenic and genotoxic compounds (Casarett et al., 2001c). Many of them have provided useful information but only a few of them are used currently in genetic toxicology. The assays used can measure the DNA damage directly or can measure the consequences of DNA damage.

When measuring the DNA damage itself, it can be done directly, through indicators such as chemical adducts or strand breaks detection, or indirectly, through measurement of biological repair processes.

Regarding detection of DNA adducts there are several techniques available but one of the first methods used and still one of the most used is the ^{32}P-postlabeling assay (Phillips, 1992). Side by side we have the determination of DNA adducts using mass spectrometry (MS). High-performance liquid chromatography coupled with tandem mass spectrometry (HPLC-MS/MS) can be a more automated analytical approach, with the advantage of being faster and providing structural confirmation of the adducts detected by ^{32}P-postlabeling (Gamboa da Costa et al., 2009; Phillips and Arlt, 2009; Singh and Farmer, 2006).

DNA breaks can be measured using the single cell gel electrophoresis assay, also called the alkaline comet assay. The comet assay is the most widely used method for measuring DNA damage in eukaryotic cells, it detects essentially SSB and alkali-labile sites at frequencies from a few hundred to several thousand breaks per cell (Langie et al., 2015). There are several alterations to the method and nowadays it can be used not only to measure damage from SSB but it allows the detection of altered purines, which are converted to breaks by the use of a specific enzyme (the digestion of the nucleoids is done by lesion-specific repair endonucleases), and it allows to differentiate between SSB and DSB (Langie et al., 2015). The basis of the comet assay is to incorporate cells into agarose embedded slides, lyse the cells to liberate their DNA, and subject them to an electrophoretic force (electrophoresis). The DNA is normally stained with a fluorescent dye for observation and image analysis. Because broken DNA fragments migrate more quickly than larger pieces of DNA, a blur of fragments (a “comet”, as can be seen it the following chapters 3 and 4) is observed when the DNA is damaged (Singh et al., 1988; Tice et al., 2000).

To study DNA damage indirectly through a biological repair mechanism, the most common repair assay in mammalian cells, *in vitro* and *in vivo*, is the Unscheduled DNA Synthesis assay (UDS), which is a measure of excision repair as described in the publication by Madle *et al.* (Madle et al., 1994).

Reviewing the assays used to measure the consequences of DNA damage there are gene mutation assays (e.g. Ames Test: Bacterial Reverse Mutation Assay) (Maron and Ames, 1983) or chromosome analysis assays (e.g. chromosome aberrations (CAs), micronuclei formation (MN) and sister chromatid exchanges (SCE)). The more common tests that measure chromosome alterations are the mammalian cytogenetic assays. Cytogenetic assays use microscopy for direct observation of the effect of interest. The goal when using cytogenetic assays is to apply methodology that permits the unequivocal visual recognition of cells that have experienced genetic damage (Casarett et al., 2001c).

One of the conventional cytogenetic tests most used is the CA assay. CAs is based on the observation of cells in metaphase for the detection of chromosomal anomalies, especially chromosome breaks and chromatid aberrations (chapter 2). Errors of DNA replication on a damaged template can lead to deletion or exchanges of individual chromatids (chromatid-type aberrations). Errors in chromosomal segregation can lead to numerical changes (e.g., monosomic, trisomic, and ploidy changes) (Casarett et al., 2001c; Natarajan and Palitti, 2008). The presence of DNA lesions which interfere with the activity of other accessory enzymes, such as topoisomerases, may also lead to chromosomal alterations visible in metaphase, such as endoreduplication (Chapter 2). The presence of endoreplicated chromosomes can lead to polyploidy, a key feature in advanced stages of cancer.

Another cytogenetic test is the SCE assay. SCE measures chromatid exchanges between chromosomes in which apparently reciprocal segments have been exchanged between the two chromatids of a chromosome. The two chromatids are visible by differential staining (Perry and Wolff, 1974) (chapters 2 and 3). Despite the convenience and responsiveness of SCE assays, data on SCE are less informative than data on chromosome aberrations. There is uncertainty about the underlying mechanisms by which SCE are formed and how DNA damage or perturbations of DNA synthesis stimulate their formation.

One of the possible mechanisms of SCE is to be a consequence of the formation of depurinating DNA lesions. When cytogenetic damage induced by glycidamide was studied by us, a strong correlation between SCE induction and DNA adduct formation was found (N7-Glycidamide-Gua) (Martins et al., 2007). Glycidamide induces SSB but, as was mentioned before, an unrepaired SSB

can be converted into a DSB. In this case, this event may take place during replication, collapsing the replication fork and leaving one free DNA end that is a substrate for homologous recombination. This newly created DSB may initiate an SCE by homologous recombination after two subsequent mitotic steps. There is growing evidence that SCE are formed from persisting SSB; for example, cells deficient in SSB repair have increased levels of SCE (Helleday, 2003; Martins et al., 2007). One rare disease is associated with high SCE background, Bloom Syndrome, an autosomal recessive disorder in humans (SCE assay is used for the diagnosis). Individuals with Bloom syndrome have a frequency of SCE about 10 times higher than the average (*https://ghr.nlm.nih.gov/condition/bloom-syndrome*). This condition is associated with mutations in the BLM (Bloom Syndrome RecQ like Helicase) gene resulting in a dysfunctional BLM protein. BLM belongs to the human RecQ family of DNA helicases that play a crucial role as genome caretakers. The BLM helicase has pivotal functions in DNA replication, recombination, and repair, namely in homologous recombination (HR) (de Renty and Ellis, 2016; Liu et al., 2014). BLM is thought to be responsible for suppressing SCE in cells, considering that SCE represent crossover recombinants (Liu et al., 2014). Thus, in summary SCE are presumed to be a consequence of errors in the replication process, perhaps involving homologous recombination.

DNA damage and cancer

The integrity of genetic material can prevent or even slow down cancer events, hereditary disease and ageing (Kramer, 1998). The highest concern regarding DNA damage is that it can induce mutations and chromosome alterations and these can lead to a possibly disease and cancer (Clancy, 2008). The knowledge that cancer is a genetic disease with multiple steps, many of which require a mutation, and that mutagens and clastogens (chromosome breaking agents) can contribute to carcinogenesis as initiators, heighten the importance of genetic toxicology as a valuable weapon for the development of biologically based dose–response models for estimating cancer risk at low environmental exposures (Casarett et al., 2001c). Over the last three decades, hundreds of chemicals have been evaluated for genotoxic effects. Genetic toxicology assays serve not only for the identification of mutagens for purposes of hazard identification but for the

characterizing of dose–response relationships and mutagenic mechanisms, both of which contribute to an understanding of genetic and carcinogenic risks.

From the various examples in the literature, two of the events associated with mutations that can initiate and propagate carcinogenesis are DNA replication in mutated regions (Giglia-Mari et al., 2011) and mutations in DNA damage repair genes in tumors cells predisposing them to accumulate even more genetic alterations (Rodrigues et al., 2013). Other important events, is the mutational alteration of proto-oncogenes which can lead to overexpression of their growth-stimulating activity, and mutations that can inactivate tumor suppressor genes, which normally restrain cellular proliferation, releasing cells from their inhibitory influence (Hanahan and Weinberg, 2000). Various human chromosome instability syndromes and DNA repair deficiencies (e.g. Bloom syndrome and *xeroderma pigmentosum*) are associated with increased cancer risk.

One of the known examples of the link between environmental-induced DNA damage and disease is skin cancer caused by excessive exposure to UV radiation and lung cancer caused by tobacco smoke (Clancy, 2008; Doll and Peto, 1981).

Another example of a link between a chromosomal alteration and cancer is chronic myeloid leukemia, associated with a balanced chromosome rearrangement which gives rise to cancer. The Philadelphia chromosome, the result of a translocation between chromosome 9 and 22 leads to the formation of the BCR/ABL fusion gene, encoding a protein with constitutive tyrosine kinase activity (Dinis et al., 2012; Salesse and Verfaillie, 2002).

Study of Natural Compounds

The main source of human exposure to natural compounds is the diet. Diet is one if not the largest source of chronic exposure, but is also the most difficult to predict a cause-effect. Some epidemiological studies performed over the years and documented by Doll and Peto (Doll and Peto, 1981) refer to dietary habits, such as ingestion of small amounts of substances that are carcinogens. One example is the ingestion of bracken fern by Japanese; individuals who eat it daily have 3 times greater risk of development esophageal cancer than those who do not. Regarding agents produced during cooking or preservation, such as benzo[a]pyrene (B[a]P) and

nitrosamines, some are considered carcinogenic. The International Agency for Research on Cancer (IARC) recently evaluated and established a relation between food intake and colorectal cancer, in the consumption of processed meat, classified as carcinogenic to humans (Group 1) and red meat classified as probably carcinogenic to humans (Group 2A) (Bouvard et al., 2015).

The World Cancer Research Foundation believes that a third of the most common cancers can be prevented through diet, maintaining a healthy weight and taking regular physical activity. This puts diet in another level, as a preventive source. Some food products seem to be less harmful than others, such as fruits and vegetables. In fact, the ingestion of vegetables and fruit was considered protective, however there are some questions that arise upon the use of certain plant products as essential oils in medicines or as drugs or in the preparation of teas.

More than 20 new drugs launched between 2000 and 2005 were originated from natural sources (e.g. terrestrial plants, terrestrial microorganisms, marine organisms, and terrestrial vertebrates and invertebrates) and a high percentage of all new chemical entities launched onto the market, if not from natural sources, were synthetic or natural mimic compounds, based on the study of pharmacophores related to natural products (Chin et al., 2006). The cancer chemotherapeutic agents paclitaxel and docetaxel, and the camptothecin derivatives, irinotecan and topotecan were responsible for approximately one third of the total anticancer drug sales worldwide in 2002 (Chin et al., 2006), which highlights the importance of such studies.

As an alternative to conventional drug therapy, natural compounds could be less cytotoxic and with fewer side effects. Human clinical trials carried out to test polyphenols reported some positive results in patients with colorectal and prostate cancer, although not as potent or as effective as their congeners synthetic drugs, but were safer and some of them were able to increase the efficacy of the drug when used in co-treatments or in chemoprevention through dietary interventions, improving chemotherapy efficacy and decreasing side effects (Armada et al., 2016).

Cancer is caused by dysregulation of as many as 500 different gene products and cancer treatments focus on the effort to develop cancer drugs for a single target, usually a single gene, gene product, or signalling pathway (Sung et al., 2012). Anticancer agents of natural origin have an advantage because they do not have a “single target” feature, but they can modulate multiple

pathways (Armada et al., 2016). They can exert their action through modulation of transcription factors, growth factors, survival factors, inflammatory pathways, invasion, and angiogenesis, which leads to inhibition or reversal of early stages of carcinogenesis.

Nevertheless, natural compounds have two sides, one dark, one bright. They can be a factor of chronic exposure which can induce mutagenesis and genotoxic events, or they can be a modulating factor in the prevention of carcinogenesis.

GENERAL INTRODUCTION

The suggestive idea that what is natural is safe has led to an increased consumption of phytochemicals in functional foods, food supplements and in complementary and alternative medicines (CAM) (van den Berg et al., 2011), especially when we talk about chronic diseases as cancer. The frequency of cancer patients that use CAM are increasing, in a metaanalysis study with more than 65 000 patients, in a total of 18 countries, from Australia, Canada, Europe, New Zealand to the United States, a prevalence of 40 % "current users" of CAM was found. The highest was in the United States and the lowest in Italy and the Netherlands. In the 1970s and 1980s the estimation was of 25 %, in the 1990s was of more than 32 % and after the year 2000 the estimation was of 49 % (Horneber et al., 2012).

The family of natural compounds presented in this study, alkenylbenzenes, consist of simple compounds that can be found in spices, food flavours, aromatic oils, perfumes, detergents, vegetables and fruits such as oranges, bananas and grapefruit juice (Chen et al., 2011; JECFA, 2009; Jordan et al., 2001). The most common spices, where we can find this family of compounds, are basil, fennel, tarragon, anise, nutmeg, coriander, chinese star anise, cinnamon, ginger, clove and black pepper (Chen et al., 2011; Lopez et al., 2015; Sangster et al., 1987). As flavours they are used in prepared foods, such as baked goods and sweets, and in alcoholic and non-alcoholic beverages (Sangster et al., 1987). About 30 alkenylbenzenes and a number of closely related compounds have been found in the essential oils of various plants (Miller et al., 1983) and two different classes of alkenylbenzenes have been identified: alkenylbenzenes with a 2,3-double

bond (Figure 1.2 - A) such as estragole, eugenol, safrole, myristicin and elemicin, and propenylbenzenes with a 1,2-double bond (Figure 1.2 - B) such as trans-anethole and β-asarone (Lopez et al., 2015). The first group of compounds (with a 2,3-double bond) can also be divided in members containing a 3,4-methylenedioxy substituent with or without an additional methoxy substituent, such as myristicin and safrole, respectively, and members containing only methoxy substituents, such as estragole and elemicin (JECFA, 2009), or with members containing one methoxy substituent, as for eugenol. Eugenol, in contrast with the other alkenylbenzenes in this group, has a free phenolic hydroxyl group (EFSA, 2009).

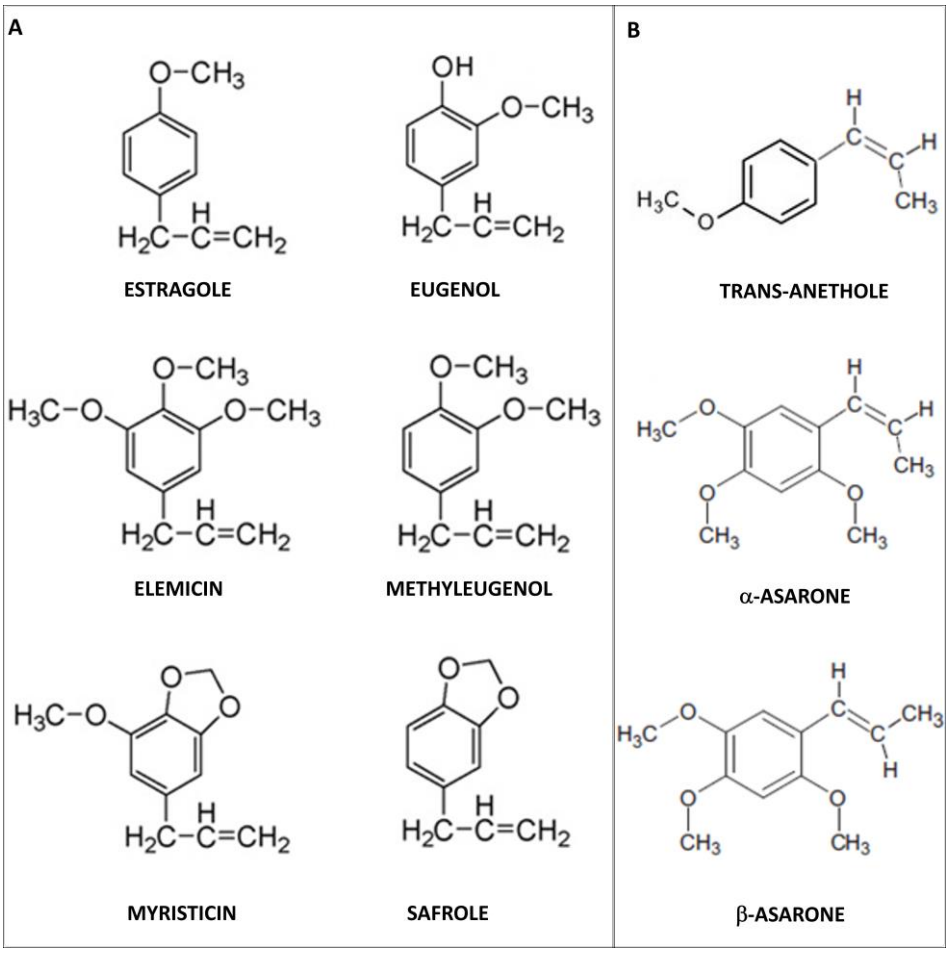

Figure 1.2 Examples of the molecular structures of the alkenylbenzenes family.

The natural sources containing these molecules have been used not only in foods but in medicines for thousands of years (Sangster et al., 1987), in essential oils, infusions, teas or even

in pharmaceutical formulations. Consequently, this family of natural compounds is present and widely used not only in Western diets (van den Berg et al., 2011) but in medicines providing a significant potential risk for human exposure to alkenylbenzenes, as some of these compounds have toxic, namely genotoxic, properties, as we shall discuss in this thesis. In some countries herbal tea beverages on the basis of bitter fennel fruits or bitter fennel oil are used for babies and infants for carminative purposes (EFSA, 2009). It's no easy task to estimate exposure when we are talking about natural compounds. Not only the sources are vast but their concentrations in each source have a significant variation. The content in alkenylbenzenes for each natural source can vary because of their geographical origin (JECFA, 2009; Randerath et al., 1993), plant maturity at harvest, harvesting techniques, storage conditions, processing, or even depending on the method used for their measurement (JECFA, 2009; Smith et al., 2002). Therefore, exposure can be unsuspected and entail toxic responses.

One example that was reported, regarding exposure to high, toxic concentrations, of an alkenylbenzene in CAM, was with a middle age woman, reported in 2012. She had an intoxication possibly caused by the alkenylbenzenes α-asarone and β-asarone. This woman consumed natural herbal products prescribed in Chinese traditional medicine and the prescription could be of more than 10 pellets/tablets per day. When the content in each pellet or tablet were analyzed they found a diverse content of asarone isomers. The dose of α-asarone ranged from 0.49 to 42.5 mg per pellet and the dose of β-asarone ranged from 142 to 645 mg and in most of the examined pellets and tablets they exceeded 115 mg, which is the maximum acceptable daily intake (Zuba and Byrska, 2012). This example takes us to a very susceptible area of natural compounds regarding their effective concentration in each preparation for use in cooking, teas or CAM.

Recently, a study was performed to determine the occurrence of fourteen flavouring compounds in 61 Dutch food products (Lopez et al., 2015). The alkenylbenzene estragole was detected in fennel tea, fish samples, Italian herbs-containing ready-to-eat savouries, herbs-containing processed vegetables and in pesto (Lopez et al., 2015). Myristicin was the most common alkenylbenzene detected in the Dutch food products, found in sixteen samples, such as beverages, nutmeg and in herbs-containing products, including meat, fish, sauces, processed

vegetables, ready-to-eat savouries and desserts (Lopez et al., 2015). Elemicin and safrole were detected only in butter with spices (Lopez et al., 2015).

In the early 1960s, one of the alkenylbenzene family member, safrole, was found to induce liver tumours in rats (Homburger et al., 1961; Howes et al., 1990; Sekizawa and Shibamoto, 1982) and was evaluated in 1976 and 1987 by IARC, which led it to be classified in Group 2B (IARC, 1976, 1987), as a possible carcinogen. In 1981 the Joint FAO/WHO Expert Committee on Food Additives (JECFA) could not allocate an ADI (Acceptable Daily Intake) due to its carcinogenicity (WHO, 1981). Safrole was voluntarily cancelled from the Environmental Protection Agency (EPA) pesticide regulation (1977) and has not been approved by the US Food and Drug Administration (FDA) for use in foods. A threshold cannot be assumed by the European Commission because of its genotoxic and carcinogenic activity, and the Committee did not establish a safe exposure limit (EU-SCF, 2002).

Due to structural similarities with safrole, some alkenylbenzenes in the '80s began to be studied regarding their hepatocarcinogenicity (Miller et al., 1983; Phillips et al., 1981; Phillips et al., 1984; Randerath et al., 1984). Miller *et al.* (Miller et al., 1983) performed a study for the detection of carcinogenicity in mice. Myristicin and eugenol (among others) were negative, but safrole and estragole were hepatocarcinogenic (Miller et al., 1983). Of the 11 naturally occurring alkenylbenzenes studied, four (safrole, estragole, methyleugenol and isosafrole) were hepatocarcinogenic, and β-asarone induces leiomyosarcomas of the small intestine in male rats (Miller et al., 1983). One of the conclusions of these studies was that despite their structural similarity, alkenylbenzenes have different modes of action, as possible genotoxic or carcinogenic compounds, probably because of the different number and type of groups in the benzene ring. Alkenylbenzenes exhibited large differences in their DNA binding activities, according to the presence of methoxy groups or a methylenedioxy group. The methylenedioxy group greatly enhanced binding of the compounds to mouse-liver DNA, and it appeared to have an optimal number of substituents for DNA binding, since mono- and di-substituted compounds bound more strongly than compounds with additional methoxy groups (JECFA, 2009; Miller et al., 1983; Randerath et al., 1984).

The range of intake of alkenylbenzenes from spices and spice oils is generally similar. Myristicin, estragole, eugenol and elemicin all exhibit a similar range of intakes, which is approximately 400–600 µg/person per day (Table 1.2). The presence of alkenylbenzenes in food sources and their content (% or ppm) in plants are also presented in Tables 1.3 and 1.4.

ESTRAGOLE ($C_{10}H_{12}O$; CAS No. 140-67-0) (IUPAC name: 1-methoxy-4-prop-2-enylbenzene ; others: methylchavicol, *p*-allylanisole, chavicol methylether, 4-methoxyallylbenzene, 1-allyl-4-methoxybenzene) is a naturally occurring flavouring agent present in a large number of aromatic plants and their essential oils (De Vincenzi et al., 2000; emeA, 2005). It is a common component of tarragon (estragon), basil (sweet basil), fennel, star anise, mace, marjoram and nutmeg (emeA, 2005; Smith et al., 2002), and a constituent of the essential oils of basil, tarragon, chervil, Mexican avocado leaves, fennel, anise and star anise (Anthony et al., 1987; Miller et al., 1983; Phillips et al., 1981).

The essential oils from this sources have been widely used in foodstuffs as flavouring agents (De Vincenzi et al., 2000) and they are present in many consumable food (processed foodstuffs) and drink products, such as fats and oils, alcoholic and non-alcoholic beverages, baked goods, frozen dairy, meat products, sweets and canned fish (Chen et al., 2011; emeA, 2005; Sangster et al., 1987; Smith et al., 2002).

In traditional medicine and as a household remedy, fennel (major source of estragole, Table 1.4) has been claimed to treat a variety of complaints especially those of the digestive system (EFSA, 2009). It has been used as antispasmodic, diuretic, anti-inflammatory, analgesic, secretomotor, secretolytic, galactagogue, eye lotion, carminative, emmenagogue, laxative, stimulant, stomachic, expectorant and antioxidant remedy and integrator (EFSA, 2009; Gori et al., 2012). Fennel powder can be used as a poultice for snake bites. In Asian cultures fennel is ingested to speed the elimination of poisons. The ancient Anglo-Saxons believed that nine herbs had special powers against illness and evil (called the nine sacred herbs) and fennel was credited with the

power to cure. Fennel was also valued as a magic herb: in the Middle Ages it was draped over doorways on Midsummer's Eve to protect the household from evil spirits (Gori et al., 2012).

The exposure to estragole and total average daily intake varies from study to study (Tables 1.2 to 1.4). Several evaluations from expert panels have been performed to assess the safety of human exposure.

In the United States, estragole is permitted for use as a synthetic flavouring substance and is approved by the FDA for direct addition to food for human consumption as a flavouring substance (EFSA, 2009; emeA, 2005). There are several evaluations regarding estragole exposure, in 1965 the Flavor and Extract Manufactures Association of the United States (FEMA) expert panel concluded that estragole is "generally recognized as safe" (GRAS) under conditions of intended use as flavouring substances in food and in 1979, the panel again evaluated the available data and reaffirmed the GRAS status of estragole for use as flavouring substance (GRASa) (Smith et al., 2002). The JECFA, in 1981, also evaluated estragole and no ADI was allocated, and made a request for additional long-term studies for evaluation of a possible carcinogenic potential (EU-SCF, 2001a, b, 2002; JECFA, 2009). Finally in 2001, the FEMA Expert panel performed a third comprehensive review of all data relevant to the safety evaluation of estragole from use as flavouring substances in food (Smith et al., 2002). Based on the evidence of a nonlinear relationship between the dose and profile effects of metabolic activation, covalent binding, and metabolism of estragole on proteins and DNA, FEMA concluded that the present exposure to estragole from spices or other sources does not pose a significant cancer risk (Smith et al., 2001). At first the average daily intake of estragole per head in the US was estimated to be about 70 µg/day (1.2 µg/kg bw/day), derived mainly from baked foods and beverages (Anthony et al., 1987; Bristol, 2011; Miller et al., 1983; Sangster et al., 1987). A new evaluation by FEMA (Smith et al., 2002), based on a conservative (i.e. high) estimate of the estragole content in various sources, predicted an estimate of the total average daily intake from consumption of essential oils to be 0.27 µg/kg bw/day and from consumption of spices in traditional foods to be 0.63 µg/kg bw/day. Assuming that only 10% of the population consumed all of the food containing estragole, the daily per capita intake would be 6.3 µg/kg bw (Smith et al., 2002). The average daily intake of estragole from total exposure (essential oils and spices) was estimated to be approximately 1.0

µg/kg bw per day, as mean daily per capita intake, assuming the same postulation as before, that only 10 % of the US population consumed foods containing estragole, the estimated daily per capita intake (consumers only) of either substance was estimated to be less than 10 µg/kg bw per day (Smith et al., 2002).

In Europe, in 2000 the Committee of Experts on Flavouring Substances (CEFS) of the Council of Europe evaluated estragole, and determined that exposure to humans could be of approximately 1 µg/kg bw based on one study completed with human volunteers and a limit of 0.05 mg/kg (detection limit) was recommended (EU-SCF, 2001a). In 2001, the EU Opinion of the Scientific Committee on Food on estragole proposed an estimated average intake (for consumers only) of 4.3 mg/day and the 97.5th percentile of 8.7 mg/day. The Committee was unable to estimate the relative contributions to total exposure to estragole from food containing herbs and spices or from the use of added flavourings (EU-SCF, 2001a). In conclusion, the scientific committee determined that "*mechanistic understandings of the biological effects of these alkenylbenzenes and their implications for human health have yet to be fully explored, and more data is needed. Thus the former are considered genotoxic carcinogens and the Scientific Committee on Food of the EU (EU-SCF) could not assume a safe exposure limit*" (EU-SCF, 2001a, b, 2002).

In 2005, the European Committee on Herbal Medicinal Products (EMEA/HMPC/137212/2005) prepared a public statement on the use of herbal medicinal products containing estragole. Their conclusions were that "*available toxicological data show that estragole is a naturally occurring genotoxic carcinogen with a DNA potency similar to that of safrole*". But they also concluded that exposure to estragole as a result of herbal medicinal products consumption (short time use in adults at the recommended posology) does not imply a significant cancer risk, although care was recommended regarding exposure to estragole in sensitive groups such as young children, pregnant and breastfeeding women. They also suggested minimized exposure by preparations for topical and external use because there are no data available on estragole absorption through the skin (emeA, 2005). These conclusions were based, essentially, on the presumption that because rodent studies have shown dose dependent toxicity, those events may be minimal in the dose range of 1-10 mg/kg bw, which is approximately 100-1000 times the anticipated human exposure of 6.3 µg/kg.bw/day proposed by Smith *et al.* (Smith et al., 2002).

Table 1.2 Intake of alkenylbenzenes.

	Source	Average Daily Intake		Others	References
		EU	USA		
Estragole	-	-	-	Considered as GRAS	*Smith et al., 2001*
	Baked foods, beverages	-	70 µg/day ~1,2 µg/kg bw per day	-	*Anthony et al., 1987*
	-	1 µg/Kg bw	-	Detection limit recommended: 0.05 mg/ Kg	*EU-SCF, 2001a*
	-	4.3 mg/day (consumers only) ~ 0.07 mg/Kg bw	-	-	*EU-SCF, 2001a*
	Essential oils	-	0.27 µg/ Kg bw per day	-	*Smith et al., 2002*
	Spices in food, essential oils	-	1 µg/ Kg bw per day 10 µg/ Kg bw per day (consumers only)	-	*Smith et al., 2002*
	Spices in food	-	0.63 µg/ Kg bw per day 6.3 µg/ Kg bw per day (consumers only)	-	*Smith et al., 2002*
	Food stuffs	8.78 µg/ Kg bw per day	-	-	*Coe, 2006*
	Dairy products, processed fruits, vegetables, nuts, seeds products or fish products.	-	-	Maximum Level acceptable when added in to food: 50 mg/ Kg	*EC, 2008*
	Non-alcoholic Beverages	-	-	Maximum Level acceptable when added in to food:10 mg/ Kg	*EC, 2008*
	Essential oils, spices	-	166 µg/ person per day Max = 510 µg/ person per day	-	*WHO, 2009*
	Bitter fennel fruits	33-263 µg/ Kg bw per day (60 Kg person) ~1.9-15.8 mg/ day	-	-	*EFSA, 2009*
	Herbal medicine products	-	-	Acceptable daily dose: 0.5 mg/person per day (50 Kg person)	*Emea, 2005*
Eugenol	-	1107 µg per day ~18 µg/ Kg bw per day	3364 µg per day ~56 µg/ Kg bw per day	ADI: 2.5 mg/ Kg bw	*JECFA, 2006*
	-	MSDI: 950 µg/ capita per day	MSDI: 3364 µg/ capita per day	Threshold of concern: 1800 µg/ person per day	*EFSA, 2009*
Elemicin	Nutmeg and mace	42.6 µg/ person per day Max = 274 µg/ person per day	29.9 µg/ person per day Maxi=173 µg/ person per day	-	*WHO, 2009*
	Baked Goods (parsley)	0.17 mg/ day	-	-	*Coe, 2006*
	Meats (parsley)	0.24 mg/ day	-	-	*Coe, 2006*
	Non-alcoholic beverages (nutmeg)	0.52 mg/ day	-	-	*Coe, 2006*
	Ice cream (nutmeg)	0.06 mg/ day	-	-	*Coe, 2006*
	Meats (nutmeg)	0.90 mg/ day	-	-	*Coe, 2006*
	Baked goods (nutmeg)	0.80 mg/day	-	-	*Coe, 2006*
Myristicin	Spices	9 mg per day	-	-	*Coe, 2006*
	Essential oils	4 mg per day	-	-	*Coe, 2006*
	Nutmeg and Mace and their essential oils	162 µg/person per day Max = 684 µg/person per day	116.2 µg/person per day Max = 465 µg/person per day	-	*WHO, 2009*
	Herbs and spices	0.0019 mg/Kg bw per day	-	-	*Al-Malahmeh, 2016*

Max = Maximum Dietary Intake Level

Table 1.3 Presence of alkenylbenzenes in food and spices.

	Natural Source	Food	Content	References
Estragole	Fennel fruits	Baked goods	130 mg/kg	*EFSA, 2009*
		Alcoholic beverages	300 mg/kg 100 mg/kg	*EFSA, 2009* *Coe, 2006*
		Fennel tea	1.5 - 2.5 g	*EFSA, 2009*
		Fats and oils	260 mg/kg	*EFSA, 2009*
		Meat products	1200 mg/kg 45 mg/kg	*EFSA, 2009* *Coe, 2006*
		Snack foods	700 mg/kg	*EFSA, 2009*
		Tarragon- or basil flavoured oil	250 mg/kg	*Coe, 2006*
		Gravies	190 mg/kg	*EFSA, 2009*
	Basil	Basil souce	710 mg/kg	*Avila et al., 2009*
	Fennel	4 cups tea	88 µg	*Lopez et al., 2015*
	-	Food stuff	0.7-5.2 mg/kg	*Lopez et al., 2015*
Eugenol	Basil	Basil souce	540 mg/kg	*Avila et al., 2009*
		Pesto souce	268 mg/kg	*Avila et al., 2009*
		Tomato sauce	27 mg/kg	*Avila et al., 2009*
Myristicin	-	Processed Food stuff	0.6-19 mg/Kg	*Lopez et al., 2015*
	Dill	Condiments	1.3 mg/Kg	*Hallstrom and Thuvander, 1997*
	Mace	Non-alcoholic beverages	27 mg/Kg	*Hallstrom and Thuvander, 1997*
	Mace	Backed goods	40 mg/Kg	*Hallstrom and Thuvander, 1997*
	Mace	Pickles (processed vegetables)	9.7 mg/Kg	*Hallstrom and Thuvander, 1997*
	Mace	Meats	13 mg/Kg	*Hallstrom and Thuvander, 1997*
	Nutmeg	Non-alcoholic beverages	4.6 mg/Kg	*Hallstrom and Thuvander, 1997*
	Nutmeg	Frozen dairy	21 mg/Kg	*Hallstrom and Thuvander, 1997*
	Nutmeg	Backed goods	34 mg/Kg	*Hallstrom and Thuvander, 1997*
	Nutmeg	Pickles (processed vegetables)	13 mg/Kg	*Hallstrom and Thuvander, 1997*
	Nutmeg	Meats	16 mg/Kg	*Hallstrom and Thuvander, 1997*
Elemicin	Nutmeg	Non-alcoholic beverages	14 mg/Kg	*Coe, 2006*
	Nutmeg	Ice cream	13 mg/Kg	*Coe, 2006*
	Nutmeg	Condiments	21 mg/Kg	*Coe, 2006*
	Nutmeg	Meats	150 mg/Kg	*Coe, 2006*
	Nutmeg	Backed goods	75 mg/Kg	*Coe, 2006*
	Nutmeg	Candy	19 mg/Kg	*Coe, 2006*
	Parsley	Baked goods	24 mg/Kg	*Coe, 2006*
	Parsley	Condiments	64 mg/Kg	*Coe, 2006*
	Parsley	Meats	62 mg/Kg	*Coe, 2006*
	Parsley	Soups	66 mg/Kg	*Coe, 2006*
	-	Butter with spices	1.3 mg/Kg	*Lopez et al., 2015*
	-	Pate	1.2 mg/Kg	*Lopez et al., 2015*

Table 1.4 Main occurrence of alkenylbenzenes in natural sources, plant or essential oils.

	Natural Source	Content Plant (ppm)/or essential oils (%)	References
Estragole	Anise	1 050 ppm 1 - 5 %	*Emea, 2005* *EU-SCF, 2001a*
	Anise scanted, Basil (*Ocimum tenuiflorum* L.)	39 950 ppm	*Emea, 2005*
	Avocado	3 - 85 %	*Emea, 2005*
	Basil	1.2 ± 0.3 mg/g 91.52 %	*Avila et al., 2009* *WHO, 2009*
	Bayrum tree (*Piper betle* L.)	1.02-4.0 % / 8 %	*Emea, 2005*
	Blue mountain, Tree (*Solidago odora Ait.*)	75 %	*Emea, 2005*
	Chervil	Up to 85 % 83.10 %	*Coe, 2006* *WHO, 2009*
	Chinese star anise	280 - 6 500 ppm / 0.6 - 6 % 5 – 6 % 6.65 %	*Emea, 2005* *Coe, 2006* *WHO, 2009*
	Cinnamon	0.29 %	*WHO, 2009*
	Cumin	30 ppm 0.05 %	*Emea, 2005* *WHO, 2009*
	Fennel	70 – 4 018 ppm / 0.8 - > 80% 3.5 – 12 % 1.5 - 5.0 % 6.21 %	*Emea, 2005* *EFSA, 2009* *Coe, 2006* *WHO, 2009*
	Garden cheroil (*Anthriscus cerefolium (L.) Hoffm.*)	75 %	*Emea, 2005*
	Giant Hyssop	555 – 12 160 ppm / 43.7 % 74 %	*Emea, 2005* *Coe, 2006*
	Hyssop	1 - 260 ppm	*Emea, 2005*
	Jamaica pepper	3 ppm	*Emea, 2005*
	Lemon balm	6.3 %	*Coe, 2006*
	Marjoram	0.4 %	*WHO, 2009*
	Myrtle	58 - 88 ppm	*Emea, 2005*
	Oregano	1.6 %	*WHO, 2009*
	Pimento	30 – 10 745 ppm	*Emea, 2005*
	Rosemary	3 %	*WHO, 2009*
	Schrubby basil (*Ocimum canum Sims.*)	52 %	*Emea, 2005*
	Sweet basil (*Ocimum basilicum L.*)	238 – 8 780 ppm / 5 – 85 % 31.21 % 20 – 89 %	*Emea, 2005* *De Vincenzi et al., 2000* *Coe, 2006*
	Sweet majoram (*Origanum majorana L.*)	96 - 550 ppm	*Emea, 2005*
	Tarragon	172 - 7000 ppm 60 – 75 % 80.02 %	*Emea, 2005* *Coe, 2006* *WHO, 2009*
	White fraxinella (*Dictamnus albus L.*)	200 - 605 ppm	*Emea, 2005*
Myristicin	Aniseed	0.82 %	*WHO, 2009*
	Celery seed	1.2 % 0.33 mg/Kg	*WHO, 2009* *Hallstrom and Thuvander, 1997* *Coe, 2006*
	Coriander seed	0.05 %	*WHO, 2009*
	Dill weed	3.2 % 2.8-7.6 % 1200 mg/Kg 2.8-7.6 % 1100 mg/Kg	*WHO, 2009* *Hallstrom and Thuvander, 1997* *Hallstrom and Thuvander, 1997* *Coe, 2006* *Coe, 2006*

	Mace	6.2 % 7 - 18 % 27000 mg/Kg	*WHO, 2009* *Hallstrom and Thuvander, 1997* *Hallstrom and Thuvander, 1997*
	Nutmeg	4 % 14 % 16.9 ± 0.6 mg/g 4-8 % 13000 mg/Kg	*Randerath et al., 1993* *WHO, 2009* *Avila et al., 2009* *Hallstrom and Thuvander, 1997* *Hallstrom and Thuvander, 1997* *Coe 2006*
	Nutmeg seeds	13.57 %	*Muchtaridi et al., 2010*
	Parsley	36 % 727 mg/Kg	*JECFA, 2009; WHO, 2009* *Hallstrom and Thuvander, 1997*
	Parsley seed	77 %	*JECFA, 2009; WHO, 2009*
Elemicin	Dill weed	0.22 %	*JECFA, 2009; WHO, 2009*
	Elemi-tree	2.5 - 10.6 %	*Coe, 2006*
	Mace	3.14 %	*JECFA, 2009; WHO, 2009*
	Parsley	1.5 % 4.6 %	*JECFA, 2009; WHO, 2009* *Coe, 2006*
	Parsley seed	8.8 %	*JECFA, 2009; WHO, 2009*
	Nutmeg	5.6 % 0.3-4-6 %	*JECFA, 2009; WHO, 2009* *Coe, 2006*
	Nutmeg seeds	1.42%	*Muchtaridi et al., 2010*
	Sassafras	1.0 %	*Coe, 2006*
Eugenol	Basil	0.8 ± 0.2 mg/g	*Avila et al., 2009*
	Betel	2.51mg/g	*IARC, 2004*
	Clove	28.5 ± 0.4 mg/g	*Avila et al., 2009*
	Nutmeg	0.6 ± 0.1 mg/g	*Avila et al., 2009*

In 2008, the European Union (EU) determined that estragole and safrole should not be added as such to food and established maximum levels for the use of substances where they are naturally present, such as flavourings and/or food ingredients with flavouring properties (EC, 2008). Estragole should not exceed 50 mg/Kg in dairy products, processed fruits (vegetables including mushrooms, fungi, roots, tubers, pulses and legumes and nuts and seeds), and fish products. For non-alcoholic beverages the maximum level should not exceed 10 mg/kg (EC, 2008).

The most recent publication on estragole evaluation was performed in 2009, in which the FAO/WHO estimates, based primarily on USA import data for spice and spice oils, a maximum dietary intake level for estragole of 510 µg/person per day, with a mean value of 166 µg/person per day (JECFA, 2009; WHO, 2009). In their "safety evaluation of certain food additives", prepared by the sixty-ninth meeting of the JECFA, they concluded that the data reviewed on the alkenylbenzenes studied (namely estragole) provide evidence of toxicity and carcinogenicity to

rodents given high doses. They refer to a lack of mechanistic understanding of these effects and their implications for human risk and those studies are needed and will have a significant impact on the assessment of health risks for alkenylbenzenes at the concentrations at which they occur in food (JECFA, 2009; WHO, 2009).

The European Food Safety authority (EFSA) estimated a much higher exposure to estragole, only from bitter fennel fruits, of 33 to 263 µg estragole/kg bw/day for a 60 kg person. The calculations were done assuming that we need to use 4.5 to 7.5 gram (3 times 1.5 to 2.5 g) of fennel fruits per day for the preparation of fennel tea, that fruits contain 5 % of essential oil, that the extraction efficiency of the essential oil is of about 25 to 35 %, and that there is 3.5 to 12 % estragole, at the end that would imply an intake of 1.9 to 15.8 mg estragole per day (EFSA, 2009). But we have to take in to account that fennel infusions are a classical decoction for nursing babies and children to prevent flatulence and colic spasms (Gori et al., 2012). Accordingly, for children the exposure can be higher, the preparation of fennel fruits tea for carminative reasons, can be used at 9 to 15 grams, although there are recommendations for short term use (less than one week), and that use is not recommended in children under 4 years of age (EFSA, 2009; EU-SCF, 2001a, b, 2002).

The Margin of Exposure (MOE) for estragole from bitter fennel fruits (preparation of fennel tea) is about 34 to 1000 (EFSA, 2009). MOE is defined as the ratio between the lower confidence limit of the benchmark dose that gives 10% extra cancer incidence (BMDL10) and the estimated daily intake (EDI) for estragole (EFSA, 2005). Rietjens *et al.* (Rietjens et al., 2010), based on an estimation of an average per capita daily intake of estragole of 0.07mg/kg bw/day (EU-SCF, 2001a) and a BMDL10 of 9–33 mg/kg bw.day calculated a MOE for pure estragole of 129–471 (Rietjens et al., 2010). As described by EFSA a MOE lower than 10.000 can be considered a priority for human risk (EFSA, 2005).

The content of estragole in food stuffs, in Europe, was assessed in at least two studies. Avila *et al.* (Avila et al., 2009) analyzed alkenylbenzenes contents in seven different food/spices samples, most of which were used in traditional cuisine and as flavorings in commercial food. Estragole was found in Basil at 1.2 mg/g and in the Basil sauce at 710 mg/Kg (Avila et al., 2009). In 2015, in another study performed to determine the occurrence of flavouring compounds in 61 Dutch food products, it was estimated that the intake of four cups per day of fennel tea would imply a

consumption of 88 µg of estragole (Lopez et al., 2015). In addition to fennel tea, estragole was detected at the range of 0.7–5.2 mg/kg in two fish samples, two Italian herbs-containing ready-to-eat savouries, two herbs-containing processed vegetables and in one pesto (Lopez et al., 2015), well below the limit stablished by the EU of 50 mg/Kg .

When we talk about exposure we should also think about "specialized consumer groups" as was cited before. Those consumers can be exposed to much higher levels of alkenylbenzene derivatives. As an example we have pesto eaters which ingest some of the highest levels of alkenylbenzenes. Fresh pesto is prepared from a large quantity of fresh sweet basil, considering that a single portion of pesto may contain up to 10 g of basil (oil content 0.5 %) and that the most commonly used basil in pesto preparation in north-western Italy, contains more than 40 % of the alkenylbenzene methyleugenol, a typical serving of pesto may provide up to 250 µg/kg bw of methyleugenol (JECFA, 2009). In the study by Lopez et al. with Dutch food, methyleugenol was detected in a range of 0.6 – 3.3 mg/kg in an industrial pesto but in a homemade pesto was at 185 mg/kg, which exceeds the maximum permitted level in food for methyleugenol (28-98 mg) (Lopez et al., 2015).

In this particular case, of home-made pesto, no regulatory limit can be applicable by the EU since in the recommendations it specifies that the maximum levels do not apply when "*a compound food contains no added flavourings and the only food ingredients with flavouring properties which have been added are fresh, dried or frozen herbs and spices."* (EC, 2008). Thus a single consumption of such foods may result in intakes 1 to 2 orders of magnitude greater than mean intakes. However, we can also argue that pesto is not consumed daily and even for these specialized consumer groups, their average lifetime intake when calculated on a daily basis may approach mean or maximum daily intake levels for non-specialized consumer groups (JECFA, 2009).

The EMEA public statement of 2005 (European Committee on Herbal Medicinal Products) regarding the use of herbal medicinal products containing estragole is currently under revision (24 November 2014; HMPC) although, since 2014, no final conclusions were published. Nonetheless they recommend that because of the generally accepted evidence of genotoxic carcinogenicity, exposure to estragole should be kept as low as practically achievable. Regarding

high exposure groups, they suggest a conservative estimate to provide at least a 10-fold increase in the limit value. They calculated an acceptable daily dose of 0.5 mg/person/day.

EUGENOL ($C_{10}H_{12}O_2$; CAS No. 97-53-0) (IUPAC name 4-Allyl-2-methoxyphenol; others: 2-methoxy-4-prop-2-enylphenol; 1-allyl-3-methoxy-4-hydroxybenzene) is essentially extracted from clove oil and marjoram but is also present in spices such as basil, cinnamon bark and leaf, nutmeg, oregano, tarragon, dill, rosemary, pimento leaf and berry, and laurel (Guenette et al., 2007).
Eugenol, among other alkenylbenzenes (e.g. methyleugenol, elemicin) was also found in the juice and essential oil of oranges before and after the trees sprayed with abscission agents at levels of 4 to 40 ppb (Miller et al., 1983) and occurs naturally in various foods and drinks such as wheaten bread, apples, ice cream, cherries, whisky and red and white wine (JECFA, 2006; Kamatou et al., 2012). As an example, in the quantification of eugenol in samples of spices and sauces normally used in traditional gastronomy, eugenol was found in the spices basil, nutmeg and clove at 0.8, 0.6 and 28.5 mg/g, respectively. In samples of sauces of basil, pesto and tomato eugenol was found at 540, 268 and 27 mg/Kg, respectively. The presence of eugenol in these samples of sauces is probably due to basil presence in their preparation (Avila et al., 2009).
Most of eugenol uses has to do with is flagrance, so eugenol is a common ingredient in cosmetics and several popular perfumes (Kamatou et al., 2012) and it is also found in clove cigarettes (Bodell et al., 1998). But the use of eugenol goes beyond its flavouring properties, as this alkenylbenzene is highly used in medicines, such as in dentistry and in Chinese medicine preparations (Guenette et al., 2007). In dentistry it is used in mouthwashes and in combination with zinc oxide to form a polymerised eugenol cement used for surgical dressings, temporary fillings, pulp capping agents and cavity liners (Kamatou et al., 2012). The pharmacological action of eugenol has to do with antiseptic and analgesic properties, by according to published results it is also suggested that eugenol can have an anti-inflammatory and anti-oxidant capability (Lionnet et al., 2010).

The estimated daily exposure to eugenol is Europe is of 1107 µg/day comparing to 3364 µg/day in the USA. The threshold of concern for this class of compounds is of 1800µg/person/day.

Eugenol is approved for its use as a flavouring agent in the European Union. JECFA in their twenty-sixth meeting evaluated eugenol and an ADI of 0–2.5 mg/kg bw was assigned (JECFA, 2006). This ADI value was proposed on the basis of the results of a 19-week study in rats. In the NTP study in 1983 a NOEL of 300 mg/kg bw per day was established (NTP, 1983), more than 16 000 and 5000 times the estimated daily exposure to eugenol from its use as a flavouring agent in Europe (18 µg/kg bw) and the USA (56 µg/kg bw), respectively. Because of that the most recent evaluation on eugenol by JECFA (JECFA, 2006) considered that eugenol would not present a safety concern at the estimated daily exposure.

In the European Union eugenol was evaluated in 2008 by EFSA (EFSA, 2009), the MSDI (Maximized Survey-derived Daily Intake) for eugenol in Europe (950 µg/capita/day) is below the threshold of concern (1800µg/person/day) of the structural class, so they concluded to be of no safety concern.

MYRISTICIN ($C_{11}H_{12}O_3$; CAS No. 607-91-0) (IUPAC name: 4-methoxy-6-prop-2-enyl-1,3-benzodioxole; others: Myristicine; 1-allyl-5-methoxy-3,4-methylenedioxybenzene; 6-allyl-4-methoxy-1,3-benzodioxole; 4-methoxy-6-(2-propenyl)-1,3-benzodioxole) is found in nutmeg, basil, anise, cinnamon, marjoram, bay, parsley, star anise and clove and is present in their essential oils. It is also used as a flavouring agent and is commonly present in beverages (e.g. cola drinks) and herb-containing products (Lopez et al., 2015; Ozaki et al., 1989; Randerath et al., 1993). In traditional and complementary medicines myristicin is used to treat rheumatism, cholera, psychosis, stomach cramps, nausea, diarrhoea, and anxiety (Ozaki et al., 1989).

Overall, exposure to myristicin in Europe occurs mainly due to its use as a flavour ingredient especially through consumption of nutmeg and mace. These spices are extensively used worldwide in many foods, and annual production is measured in thousands of tons. Certain prepared foods, such as puddings, sweet sauces, and baked goods, contain nutmeg at a level of 0.3% by weight (JECFA, 2009; Randerath et al., 1993). Avila *et al.* (Avila et al., 2009), quantified the level of alkenylbenzenes in seven different samples (basil, nutmeg, clove, anise, cassia, pesto sauce and tomato sauce) most of which used in traditional gastronomy and myristicin was found in nutmeg at 16.9 mg/g.

The Committee of experts on flavouring substances of the Council of Europe (Coe, 2006) reported an average consumption of about 9 mg/day of myristicin from spices, and about 4 mg/day from essential oils. In 2009, the FAO/WHO estimate that the dietary exposure per capita of the European population to myristicin, via the consumption of nutmeg and mace and its oil were on average 162 μg/day, the lower limit of 3 and higher limit of 684 μg/day (JECFA, 2009; WHO, 2009). At present, myristicin is not regulated by the EU (EC, 2008; Lopez et al., 2015).

Al‑Malahmeh (Al-Malahmeh et al., 2016) performed a calculation of a MOE for myristicin based on the existent data for safrole. Tumor data for myristicin is scarce so the authors extrapolated from the results presented by Van den Berg (van den Berg et al., 2011) for safrole, indicating that 1'-sulfoxy metabolites of myristicin in human liver is comparable (1.1-fold higher) to that of safrole. On the basis of these considerations, and considering an estimated daily intake for myristicin of 0.0019 mg/kg bw per day from use of herbs and spices, the MOE for myristicin was predicted to be 1000–2684 (Al-Malahmeh et al., 2016). As mentioned before, a compound is considered to be of priority for risk management actions and a concern for human health when the MOE is lower than 10,000.

However exposure to myristicin is not restricted to nutmeg, in a recent study myristicin was detected in sixteen samples of 61 Dutch food products, including meat, fish, sauces, processed vegetables, ready-to-eat savouries and desserts, ranging from 0.6 to 19 mg/kg (Lopez et al., 2015), being one of the most common alkenylbenzenes detected in the Dutch food products (Lopez et al., 2015).

Nutmeg oil has been reported to contain about 4% myristicin and about 0.6% safrole, but its composition varies depending on geographical origin (Randerath et al., 1993). Something to also take in to account is that nutmeg is used as a low cost alternative to recreational drug and some intoxication and even fatal cases were attributed to myristicin poisoning (Demetriades et al., 2005; Hallstrom and Thuvander, 1997; Stein et al., 2001). The ingestion of nutmeg to "get high" can be of approximately 5 g, corresponding to 1–2 mg myristicin/kg bw but 6 to 7 mg/kg bw may be the dose required to cause psychopharmacological effects in humans (Hallstrom and Thuvander, 1997). A 13 year old female ingested 15 to 24g of nutmeg (Sangalli and Chiang, 2000) and an 18 year old female ingested 50 g of commercially available grated nutmeg blended into a

milkshake and drank three quarters of the amount (Demetriades et al., 2005). This attempts of "nutmeg induced euphoria" is said to have been common in the ''hippie culture'' of the 1960s and 1970s. It has also been reported in drug addicts, prisoners, adolescents, and college students where it is regarded as an affordable alternative to limited supplies of ethanol and recreational drugs (Demetriades et al., 2005). The ingestion of grated or whole nutmegs are often mixed with coffee, vodka or other alcohol, and concurrent use with cannabis (Demetriades et al., 2005). A fatal case, after nutmeg ingestion, was reported with a 55-year-old woman and myristicin was detected at 4μg/mL in the postmortal serum. Death was presumably caused by a combined toxic effect with flunitrazepem. Cases of 20 to 80 g ingestion have been reported, the equivalent to 280-1100 mg/kg exposure (Stein et al., 2001).

ELEMICIN ($C_{12}H_{16}0_3$; CAS No. 487-11-6) (IUPAC name: 1,2,3-trimethoxy-5-prop-2-enylbenzene; others: 1-a!lyl-3,4,5-trimethoxybenzene; 3,4,5-trimethoxyallylbenzene). From the group of alkenylbenzenes studied in this thesis, elemicin is the one with less information and only limited toxicological data are available. Elemicin is not regulated by the EU (EC, 2008; Lopez et al., 2015) and human exposure to elemicin is essentially from nutmeg, mace, tarragon, parley seed oil, parsley and sassafras (JECFA, 2009; van den Berg et al., 2012), and from products made thereof including plant food supplements (van den Berg et al., 2012). Recently, elemicin was detected in butter with spices at 1.3 mg/kg and in pate at 1.2 mg/kg (Lopez et al., 2015). Elemicin was also found in the juice of oranges from trees sprayed with abscission agents (Miller et al., 1983).
FAO/WHO estimate, based primarily on EU import data for nutmeg and mace, a maximum dietary intake level for elemicin of 274 μg/person per day, with a mean value of 42.6 μg/person per day. In the USA the exposure to elemicin from spice and spice oils was estimated at 173 μg/person per day, with a mean value of 29.9 μg/person per day (JECFA, 2009; WHO 2009). Elemicin contribution to the overall intake from all sources seems to be minor compared to estragole and myristicin (JECFA, 2009). Because of some equivocal results regarding genotoxicity and carcinogenic studies, and the high concentrations tested in each assay in 2008, JECFA (JECFA, 2008) concluded that further research should be conducted to assess the potential risk to human health from low-level dietary exposure to alkoxy-substituted allylbenzenes present in foods and

essential oils and used as flavouring agents, such as for elemicin and all others described in this thesis.

A MOE factor was predicted by Van den Berg (van den Berg et al., 2012) for elemicin, and the results obtained demonstrated that elemicin when compared to other alkenylbenzenes, such as estragole and methyleugenol, have a lower priority for risk management. The MOE's calculated were based on elemicin content in nutmeg-containing plant food supplements (MOE = 80-3000), essential oils (MOE = 40000-1000000) and from spices, food and essential oils (MOE = 2000-4000). As mentioned before, a compound is considered to be of priority for risk management actions and a concern for human health when the MOE is lower than 10,000. Thus, from this study MOE's calculated for plant food supplements (3000) and spices, foods and essential oils (4000) should be a priority for risk management although they were higher than for other alkenylbenzenes. But when MOE is calculated based on essential oils no priority is accounted.

METABOLISM AND PHARMACOKINETICS OF ALKENYLBENZENES

Alkenylbenzenes were found to be biotransformed in the liver, in a very similar manner. A number of shared biotransformation pathways exist for this group of substances (JECFA, 2009). Studies performed in rodents indicate that the metabolism and pharmacokinetics of this group of compounds are strongly dose dependent (JECFA, 2009) .

Their metabolism can be characterized by three main metabolic pathways and there is evidence that the pathway addressed is dose-dependent. At low doses (<1-10 mg/Kg bw) the compound may be safely disposed of by metabolism, whereas at higher doses (~ 1000 mg/Kg bw) these pathways may be saturated, thereby throwing emphasis on other, formerly minor, routes giving rise to toxic metabolites (Anthony et al., 1987). At low doses, ring substituents (e.g. methoxy) are metabolised and at higher concentrations biotransformation switches additionally to the oxidation of the allyl side chains. In mice and rats, as the dose is increased (0.05-1000

mg/Kg body weight (bw)), the extent of the *O*-demethylation pathway (detoxification route) decreases while 1´-hydroxylation increases (toxication route). Another toxication route is via epoxidation of the allyl side chain, but it appears to be not as significant as activation via the 1´-hydroxylation pathway. Therefore, alkenylbenzenes, although they are not electrophilic, can form reactive electrophilic intermediates via biotransformation that can lead to the ultimate carcinogenic compound (Anthony et al., 1987; Guenthner and Luo, 2001). Eugenol, in contrast with the other alkenylbenzenes in this group, has a free phenolic hydroxyl group. This group can be seen as an active group for ready conjugation with glucuronic acid or sulphate and facilitating is excretion in the urine. Estragole, elemicin and myristicin are more susceptible to oxidation in the propenyl chain (EFSA, 2009).

Regarding alkenylbenzenes' biodistribution and half-life in the body there are limited studies in spite of the high importance of these studies (Guenette et al., 2007). Based on metabolism experiments and data from pharmacokinetic studies it can be extrapolated that this group of substances undergoes rapid and essentially complete absorption following oral Intake (JECFA, 2009). Based on studies in rats and humans, these substances are highly bioavailable following the consumption of spices containing these constituents (Beyer et al., 2006).

Pathway I: *O*-demethylation

This pathway (Figure 1.3, left side) seems to be the most predominant at low doses in rodents, and regarding human exposure it can be the most prevalent. In this pathway the *p*-methoxy substituent or one of its metabolites undergoes *O*-demethylation yielding the corresponding phenolic derivative that can be excreted as sulphate or glucuronic acid conjugate (Smith et al., 2002).

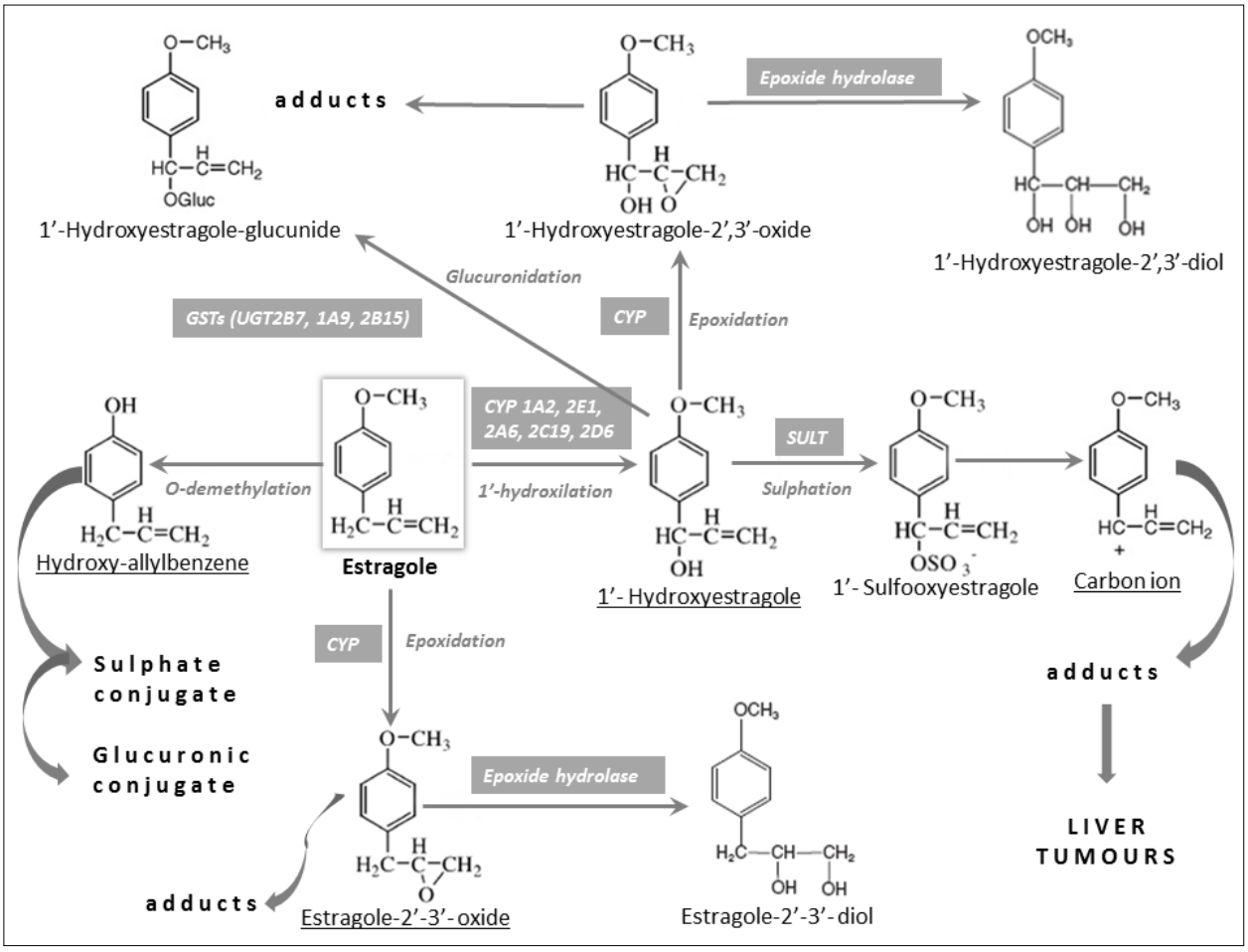

Figure 1.3 Possible metabolic activation pathways for estragole and phase I and II enzymes. Figure adapted from *Chen et al., 2011* and *Rietjans et al., 2005.* (CYP=Cytochrome P450 ; GST= Glutathione-S-Transferase ; SULT= sulfotransferase)

Pathway II: Epoxidation

Epoxidation can also occur in this family of compounds (Figure 1.3, down). The epoxidation occurs at the allyl side chain yielding the 2´-3´-oxide. This epoxide formed is readily detoxified by epoxide hydrolases (EH) to form the diol or via glutathione conjugation by glutathione transferase (GST) (Smith et al., 2002). The corresponding 2´-3´-diol, resulting from the epoxide hydration, was found in small quantities in the urine of rats and mice (Anthony et al., 1987). Therefore, epoxide metabolites of alkenylbenzene analogues can be formed *in vivo*. Guenthner et al. (Guenthner and Luo, 2001) found that these epoxides can be sufficiently electrophilic to readily form covalent adducts with proteins and DNA bases *in vitro*, demonstrating that this

pathway is a potential genotoxic bioactivation pathway. However, regarding epoxide formation, not only are they metabolized much more rapidly than they are formed in the rat liver, but the general rate of epoxide hydrolysis is much greater in human liver than in rat liver. This may suggest a high margin of protection from DNA covalent adduct formation, as for genotoxicity resulting from this pathway (Guenthner and Luo, 2001).

In human livers, epoxide hydrolase activity is subject to a wide variation. At least a 20-fold interindividual range is seen between the highest and lowest observed activities. Even so, humans with the lowest activities demonstrate epoxide hydrolase activity at least equivalent to that seen in rat liver (Guenthner and Luo, 2001). Therefore, it suggests a degree of protection even in those human individuals with the lowest epoxide hydrolase activity, with a greater margin of safety for those with higher activities.

Pathway III: 1'-hydroxylation

This pathway (Figure 1.3, right) is considered the main bioactivation route for alkenylbenzenes. It involves the hydroxylation of the 1´-carbon atom of the side chain (1- hydroxylation), which is both benzylic and allylic, followed by the sulphation of the resulting alcohol (Anthony et al., 1987; Miller and Miller, 1983; Randerath et al., 1984). Further, this unstable sulphate ester readily loses the *O*-sulphate moiety, leaving an electrophilic carbonium ion, resulting in reactive intermediates (carbonium ion or quinonium cation) capable of reacting with nucleotides or proteins and forming DNA adducts (Chen et al., 2011; Howes et al., 1990). This pathway is considered to produce the proximate hepatotoxic and hepatocarcinogenic agent in rodents.

ESTRAGOLE, as other alkenylbenzenes, has a dose–dependent metabolism that was demonstrated in rodents and in humans (Anthony et al., 1987; Sangster et al., 1987; Zangouras et al., 1981). Studies suggest that at low dose levels estragole is rapidly cleared from the system, with *O*-demethylation being the major metabolic route but as the dose increases, the extent of *O*-demethylation falls and other pathways, notably 1'-hydroxylation, come into prominence (Anthony et al., 1987).

The estragole metabolite 1'-hydroxyestragole is the supposed proximate carcinogen requiring the involvement of cytochromes P450 but there is also the involvement of sulfotransferases (SULTs) by converting 1'-hydroxyestragole to 1'- sulfooxyestragole, which produces the ultimate carcinogenic species, very unstable in an aqueous environment, as well as carbocation upon breakdown of 1'- sulfooxyestragole and release of the sulfate group (Anthony et al., 1987; Rietjens et al., 2005).

Glucuronidation instead of sulfation of 1'- hydroxyestragole by UDP-glucuronosyltransferase 2B7 or 1A9 provides a possible detoxifying alternative route (Rietjens et al., 2005).

Another possible route of metabolism of estragole is the oxidation of the side chain at the 2´-3´double bond (epoxidation) (Anthony et al., 1987; Chen et al., 2011; Miller et al., 1983). In feeding studies with rodents or isolated hepatocytes and in the mouse liver following administration of estragole, only trace amounts of epoxide metabolites were found, and the same happened for the formation of adducts (Chen et al., 2011; Guenthner and Luo, 2001). Possible because of a rapid detoxification by epoxide hydrolases and glutathione S-transferases (GSTs) (Chen et al., 2011; Rietjens et al., 2005).

However, following perfusion of rat livers with estragole, dihydrodiol metabolites (presumably representing end products of the epoxidation pathway) were readily formed and recovered at significant levels in the urine of animals fed with estragole and accounted for up to 30% of the total metabolic clearance of estragole (Guenthner and Luo, 2001). In this study the epoxidation pathway was quantitatively equivalent to the 1´-hydroxylation pathway in terms of the overall metabolism (Guenthner and Luo, 2001).

In summary, studies performed in the late 70's and in the 80's with rodents, suggest that, when high doses (~1000 mg estragole/kg bw) are given the 1´- hydroxy metabolite is excreted at a much higher rate compared with low doses (>0.05 mg estragole/kg bw). In groups of rats and mice receiving ^{14}C-estragole in doses of 0.05, 5, 500 and 1000 mg/Kg, urinary levels of 1´-hydroxyestragole were measured. 1´-hydroxyestragole formation reflected a dose-dependent increase: in the rat it was 0.9 % at 0.05 mg/kg bw, 3.6 % at 5 mg/kg bw, 7.5 % at 500 mg/kg bw and 8.0% at 1000 mg/kg bw (Zangouras et al., 1981).

In another dose-dependent kinetic study, oral administration of ^{14}C estragole to female Wistar rats at approximately the same doses as before (0.05 to 1000 mg/kg bw), the majorities of the low doses (0.05– 50 mg/kg bw) were eliminated as ^{14}C-labelled carbon dioxide in expired air (average of 55% on day 1 and 2.7% on day 2) and urinary elimination accounted for, on average, 32.4% of the total radioactivity after 2 days. At the higher dose levels (500 and 1000 mg/kg bw), elimination of radioactivity via expired air was less (average of 29% on day 1 and 17% on day 2), and urinary elimination was greater (average of 30% on day 1 and 29% on day 2), indicating a changeover in metabolism and elimination (Anthony et al., 1987).

Drinkwater et al., after i.p injection of ~274.17 mg/Kg bw of outbred male CD -1 mice, recovered approximately 25% of estragole in the urine after 24 hours as a conjugate (presumably the glucuronide) of 1'-hydroxyestragole (Drinkwater et al., 1976).

The results have shown that the pathways of metabolism and excretion of estragole are markedly dependent upon the dose administrated. As the dose is increased major changes occur in the relative importance of the major routes of metabolism and excretion (Sangster et al., 1987).

It can be predicted that at low dose levels, humans, mice and rats show a similar tendency to metabolise alkenylbenzenes derivatives by *O*-demethylation (Smith et al., 2002).

Regarding the metabolism of estragole in humans, one study with human volunteers was performed. Two humans were fed with 100µg of ^{14}C-methoxy-labelled estragole in a gelatin capsule (1.5 µg/kg bw). Estragole was readily absorbed from the gastrointestinal tract, and more than 35% was eliminated in the urine after 8 h, 49.4% after 24 h and 61.2% after 48 h. More than 11% of the ^{14}C was eliminated in expired air after 8 h. Approximately 70% of the dose was recovered within 48 h (Sangster et al., 1987). The main identified metabolites included those derived from *O*-demethylation and oxidative degradation of the allyl side chain (i.e. 4-methyoxyhippuric acid, the glycine conjugate of 4-methoxycinnamic acid, and 4-methoxyphenyllactic acid). Urinary 1´-hydroxyestragole accounted for an average of 0.3% of the total dose (Sangster et al., 1987).

Other data regards studies in human liver microssomes to determine bioactivation and detoxification enzymes. It seems that the most important cytochrome P450 involved in the bioactivation of estragole to its proximate carcinogen 1'-hydroxyestragole is P450 1A2. Kinetic

studies in human livers shown that at more physiologically relevant concentrations of estragole (100 - 500µM) P450 1A2 and 2A6 are the most important enzymes for bioactivation. Other CYPs may be involved, CYP 2E1, 2C19 and 2D6, but only at relatively high estragole concentrations (200 - 1000µM) (Jeurissen et al., 2007). The main detoxification route of 1'-hydroxyestragole is by glucuronidation mainly by conjugation with uridine diphosphate glucuronosyltransferases UGT2B7, UGT1A9 and UGT2B15 (Iyer et al., 2003). In rodents elimination of 1'-hydroxyestragole by glucuronidation was about 30% of urinary metabolites (Iyer et al., 2003).

EUGENOL. Regarding the metabolism of eugenol it is very similar to other alkenylbenzenes. The difference remains in the free hydroxyl group that allows it to undergo conjugation reactions directly, and thus is likely to be detoxified more efficiently. Nevertheless, eugenol can suffer biotransformation to electrophilic quinone methides, so it can potentially form oxidative base damage and DNA adducts (Bodell et al., 1998). Quinone methides are expected to combine rapidly with cellular nucleophiles (Bolton et al., 1995). During metabolism, if a quinone methide is produced it can induce the production of ROS and enter into conjugation reactions with the reduced glutathione (GSH) (Bolton et al., 1995). Quinone methides differ structurally from quinones, as one of the carbonyl oxygens is replaced by a methylene or substituted methylene group. This substitution results in a more reactive electrophile and in a reduced capacity for redox chemistry. Consequently, reactions of quinone methides in biological systems are characterized by non-enzymatic Michael additions at the exocyclic methylene carbon generating benzyl adducts of peptides, proteins and nucleic acids (Bolton et al., 1995). Studies using liver microsomal activation systems demonstrated that eugenol can be oxidized to intermediate(s) that react with microsomal proteins and reduced GSH to form adducts (Bodell et al., 1998). Incubating rat hepatocytes with eugenol results in loss of intracellular GSH before the onset of cell death, accompanied by the formation of the eugenol glutathione conjugates and covalent binding to cellular protein. These events indicate that eugenol-quinone methide is formed in hepatocytes and suggests that this electrophile is responsible for eugenol-mediated cytotoxicity (Bolton et al., 1995). In a related structure-reactivity study they further showed that replacement of the allyl substituent in eugenol with a propyl group, markedly lowered the extent of liver

damage. These data may reflect differences in the cytochrome P450 mediated formation and/or reactivity of the corresponding quinone methides (Bolton et al., 1995).

Studies regarding quantification of phase-I and phase-II biotransformation enzymes activities were completed using male Wistar rats treated by gavage with eugenol (250, 500 or 1000 mg/kg body weight) during a period of 10 days. Eugenol had no significant increase on total cytochrome P-450 content in liver microsomes but preferentially induced phase II biotransformation enzymes, such as GSTs (Rompelberg et al., 1993). From the results of this study it appears that eugenol is a more effective inducer of phase-II enzymes than of cytochrome P-450 enzymes (Rompelberg et al., 1993). In a further study with humans, the same authors could not correlate their assumptions. After consuming 150 mg/day of eugenol for 7 days there were no indications for an induction of phase-II enzyme activities. In contrast, a significant decrease in levels of GST α in plasma was found indicating that α-class GSTs in liver may be decreased due to protection against background damage of liver cells by eugenol or due to GST-inhibition by eugenol (Rompelberg et al., 1996a). Therefore, oral intake of eugenol at concentrations near the established ADI of 150 mg/day but much higher than the estimated daily exposure to eugenol in Europe of ~1.1 mg/day does not modulate glucuronidation and sulphation capacity in humans (Rompelberg et al., 1996a). However, α-class GSTs are quantitatively important for the detoxification of many compounds via human liver, and the findings of a possible decrease of α class GST levels in plasma after eugenol consumption may be important for future considerations about eugenol exposure and co-exposure with other drugs and toxic effects.

Guénette *et al.* (Guenette et al., 2007) studied eugenol's pharmacokinetics in blood of Sprague-Dawley rats following gavage administration (40 mg/kg). Concentrations of eugenol in blood and plasma peaked rapidly following oral administration. Mean T 1/2 values of eugenol in plasma and blood were long (14.0 h and 18.3 h, respectively). Following an initial rapid decline in plasma, a secondary peak occurred at approximately 4 h postdose (Guenette et al., 2007). Since only two metabolites of eugenol were identified in urine of male Sprague–Dawley rats, the glucuronide and sulfate conjugates (Guenette et al., 2006) the authors state that the sudden increase in plasma concentration may be associated to enterohepatic recirculation of the drug. Therefore,

in conclusion eugenol in rats have a long elimination half-life, suggesting that some level of accumulation can occur after repeated oral administrations (Guenette et al., 2007).

Lionnet *et al.* (Lionnet et al., 2010) studied the biodistribution of eugenol in the brain and lumbar spinal cord, in Sprague-Dawley rats, following oral administration of eugenol (40 mg/kg) for 30 consecutive days. Eugenol accumulated mainly in the spinal cord when comparing to the brain, and the authors suggest that this difference could be related to the distribution of P-glycoproteins (P-gps) or because eugenol, being highly lipophilic, tends to have a different distribution depending on the lipid content between the two areas (the lipid content in the spinal cord is twice the brain content) (Lionnet et al., 2010). For that reason eugenol seems to have an accumulation in the Central Nervous System (CNS) but without signs of ill effects and without visible lesions at the level of the central nervous system as well as other organs, suggesting that little toxicity occurs (Lionnet et al., 2010).

In summary, studies in rats suggest a possible accumulation of eugenol after repeated oral administrations with a possible accumulation in the CNS without visible toxicity.

Regarding the possibility of the interaction of eugenol with membrane proteins, e.g. P-gps, there are some studies that correlate eugenol with transporter proteins; eugenol seems to improve colchicine's oral bioavailability (Shen et al., 2011), reduces the expression of multidrug resistance protein 2 (MRP2) (Young et al., 2006) and enhances penetration in the transdermal drug delivery system (Zhao and Singh, 1998). Eugenol has been studied as a penetration enhancer for drugs in gel formulations (Lahoti, 2010; Pokharkar et al., 2011).

MYRISTICIN has been less well studied than estragole or other alkenylbenzenes, and there is only limited data available, most of them regarding studies with nutmeg. Myristicin was studied in rodents, in the 70s (Kamienski and Casida, 1970). Myristicin was administered by stomach tube to male Swiss-Webster mice at a concentration of 5 µmol/kg bw (approximately 0.60 mg/kg bw) and 48 hours later myristicin content was analyzed by expired carbon dioxide and 73% of the radioactivity was recovered from administered myristicin presumably arising from *O*-demethylenation metabolism. 15% and 3% of the radiolabelled myristicin were recovered from

the urine and faeces, respectively. Less than 2.5% of the radioactivity was detected in the liver (JECFA, 2009).

Myristicin can undergo two possible main metabolism routes, demethylenation and hydroxylation. The metabolites of myristicin were identified in the rat but also in human urine from a nutmeg abuser. In the human urine myristicin metabolites were once and twice hydroxylated at the side chain (dihydroxy myristicin) or demethylenated (demethylenyl myristicin), without detection of the main molecule (Beyer et al., 2006). Thus, the possible metabolic pathways for myristicin could be postulated as: hydroxylation of the side chain to the corresponding 1-hydroxy metabolites, bis-hydroxylation of the side chain to the corresponding 2,3-dihydroxy metabolites and demethylenation that could be followed by a methylation. Demethylenation seems to be the main metabolic step for myristicin and all metabolites were partly excreted as glucuronides and/or sulfates (Beyer et al., 2006).

From the Bioactivation pathways known for others alkenylbenzenes, 1'-hydroxylation of the alkene side chain to yield the 1'-hydroxy metabolite seems to work also for myristicin, and all detoxification (conjugation with glucuronic acid) steps or bioactivation (1'-sulfoxymyristicin metabolite) steps are similar (Drinkwater et al., 1976; Ishii et al., 2011; Miller et al., 1983; Phillips et al., 1984; Randerath et al., 1984; Wiseman et al., 1985; Wiseman et al., 1987; Zangouras et al., 1981)**.** But 1'-hydroxymyristicin formation in the liver seems to be overestimated in rats in relation to humans, 1'-hydroxymyristicin metabolite in liver is at most 1.8 fold higher in rat than in human and limited for the ultimate carcinogenic metabolite 1'-sulfoxymyristicin to (2.8–4.0)-fold higher in human (Al-Malahmeh et al., 2016).

Because myristicin is one of the main components of nutmeg, and nutmeg is used as an alternative to recreational drugs, there is some data in humans regarding predisposition of the compound in the body. In a fatal case of a 55-year-old woman after ingestion of an unknown quantity of nutmeg 4 µg/ml of myristicin was detected in the postmortal serum. In another case of nutmeg intoxication caused by ingestion of approximately 280 to 420 mg/ml of nutmeg powder, myristicin blood level was of 2 µg/ml measured 8 h after ingestion (Stein et al., 2001). Myristicin was studied in human volunteers in the form of an alcoholic drink. Twenty grams of the myristicin solution (5 mg/g) was consumed by each volunteer 1 h before blood sampling. The

concentration of myristicin detected in different human serum samples were from 17.6 µg/g to 33.25 µg/g (Dawidowicz and Dybowski, 2013).

These studies show that myristicin is one of the main active molecules in nutmeg. Concentration of myristicin after the administration of nutmeg seed essential oil in mice showed that the most concentrated compound in the plasma was myristicin. Half an hour after the addition of 1 mL/cage of nutmeg seed oil, the plasma concentration of myristicin was 3.7 µg/mL; one and two hours after the addition, the blood levels of myristicin were 5.2 µg/mL and 7.1 µg/mL, respectively. For that reason the authors correlated myristicin, as for 4-terpineole and safrole, to locomotor-inhibiting properties in mice (Muchtaridi et al., 2010).

Some of phase I enzymes were also studied for myristicin and the cytochrome P450 1A1/2 seems to be the most important for the metabolism of myristicin. Myristicin enhances the expression of P450 1A1/2, 2B1/2 and 2E1 in rat livers (Jeong and Yun, 1995) and P450 1A1/2 was also induced in mouse hepatoma - Hepa-1 cells (Jeong et al., 1997).

Regarding induction of GSTs, myristicin seems to be an active inducer of GST activity and an effective inhibitor of B[a]P-induced tumorigenesis in mice. Stimulation of GST activity by myristicin could be a major mechanism for its inhibition of B[a]P or other carcinogens that may be detoxified in the same manner (Zheng et al., 1992).

ELEMICIN, being one of the main ingredients of the volatile oil of nutmeg (Beyer et al., 2006) and because seeds of nutmeg are used for their possible psychotropic effects, elemicin was studied as one of the possible active psychotropic constituents of nutmeg. In the first studies regarding the psychotropic effects caused by nutmeg some authors attributed it to the metabolic formation of amphetamine derivatives from elemicin, safrole or myristicin. But that theory fell apart because metabolically it was a reaction difficult to occur and when they tried to detect the metabolites in the urine of human, in cases of nutmeg abuse, or rats, neither such amphetamine derivatives nor the main nutmeg ingredients could be detected (Beyer et al., 2006). Metabolites of elemicin were identified in rat urine and in the human urine of a nutmeg abuser (Beyer et al., 2006). Elemicin was *O*-demethylated at 2 positions followed by side chain hydroxylation in rats. In the human urine sample, the metabolites of elemicin identified were *O*-demethylelemicin and

O-demethyldihydroxyelemicin. Based on the metabolites identified as described above, the possible metabolic pathways based on P- 450 catalyzed conversions for elemicin wouldn't be much different for the ones found for other alkenylbenzenes: hydroxylation of the side chain to the corresponding 1-hydroxy metabolites, bis-hydroxylation of the side chain to the corresponding 2,3-dihydroxy metabolites and *O*-demethylation followed by side chain hydroxylation. Side chain hydroxylation seems to be the main metabolic step for elemicin (Beyer et al., 2006). All metabolites were partly excreted as glucuronides and/or sulfates (Beyer et al., 2006). The *O*-demethylation pathway lead to compounds that can be conjugated and excreted in the urine as the sulfate or glucuronic acid. 2',3'-epoxides can also be formed arising after epoxidation of the alkene side chain (van den Berg et al., 2012), but as was stated before although they are able to form DNA adducts *in vitro*, epoxides when formed *in vivo* are rapidly detoxified by glutathione S-transferases and epoxide hydrolases.

Differences in metabolic activation of elemicin in rat and human were predicted using a model based on physiologically-based kinetic (PBK) developed by Van den Berg (van den Berg et al., 2012). The results predicted that the differences between rat and human in what regards the formation of the proximate carcinogenic metabolite 1'-hydroxyelemicin and the ultimate carcinogenic metabolite 1'-sulfoxyelemicin are limited, although there were indications for a species-dependent difference in the bioactivation of elemicin in what regards 1'-sulfoxymetabolites. The author also compared the level of bioactivation for estragole and methyleugenol with elemicin in male rat and human liver and the results reveal that the formation of the proximate and ultimate carcinogenic metabolites of the three alkenylbenzenes of interest in rat is predicted to be comparable within an order of magnitude.

GENOTOXICITY AND MUTAGENICITY

In vitro genotoxicity and mutagenicity assays for this family of compounds are limited and most of them were negative or gave equivocal results. From the *in vivo* exposure data, there are potentially toxic intermediates, such as 1´-hydroxy metabolite and epoxides at the 2´-3´double bond (Rietjens

et al., 2005) thus in several studies the metabolites were also studied for their mutagenicity and/or genotoxicity.

Table 1.5 Genotoxicity and mutagenicity tests performed with the alkenylbenzenes estragole, eugenol, elemicin and myristicin and their metabolites 1'-hydroxy and 2'-3'-oxide.

			- S9	References	+ S9	References
ESTRAGOLE	Ames test	*Salmonella typhimurium* TA 1535	-	*Sekizawa and Shibamoto, 1982*	-	*Sekizawa and Shibamoto, 1982*
		S. typhimurium TA100	~ -	*Swanson et al., 1979* *Sekizawa and Shibamoto, 1982*	+ -	*Swanson et al., 1979* *Sekizawa and Shibamoto, 1982*
		S. typhimurium TA 98	- -	*Sekizawa and Shibamoto, 1982* *Swanson et al., 1979*	- -	*Sekizawa and Shibamoto, 1982* *Swanson et al., 1979*
		S. typhimurium TA 1538	-	*Sekizawa and Shibamoto, 1982*	-	*Sekizawa and Shibamoto, 1982*
		S. typhimurium TA1537	-	*Sekizawa and Shibamoto, 1982*	-	*Sekizawa and Shibamoto, 1982*
	rec-assay	*Bacillus subtilis* DNA repair test	-	*Sekizawa and Shibamoto, 1982*	N	N
	reversion test	*Escherichia coli* WP2 uvrA	-	*Sekizawa and Shibamoto, 1982*	-	*Sekizawa and Shibamoto, 1982*
	UDS	Rat hepatocytes	+ + + +	*Howes et al., 1990* *Muller et al., 1994* *Chan and Caldwell, 1992* *Nesslany et al., 2010*	N	N
		Rat (in vivo) hepatocytes	+ +	*Muller et al., 1994* *Nesslany et al., 2010*	N	N
	MN	*Mouse (in vivo)*	-	*NTP, 2008*	N	N
1'-hydroxy	CA	*V79*	-	*Muller et al., 1994*	-	*Muller et al., 1994*
		Rat hepatocytes	-	*Muller et al., 1994*	N	N
	Ames test	*S. typhimurium* TA1535	+	*Swanson et al., 1979*	N	N
		S. typhimurium TA100	+	*Swanson et al., 1979*	+	*Swanson et al., 1979*
		S. typhimurium TA98	- +	*Swanson et al., 1979* *Swanson et al., 1979*	N	N
2',3'-oxides	Ames test	*S. typhimurium* TA1535	+	*Miller et al., 1983*	N	N
		S. typhimurium TA100	+	*Miller et al., 1983*	N	N
		S. typhimurium TA98	-	*Swanson et al., 1979*	-	*Swanson et al., 1979*

Cont.			- S9	References	+ S9	References
EUGENOL	Ames test	*S. typhimurium* TA 1535	- -	*Swanson et al., 1979* *Sekizawa and Shibamoto, 1982*	- -	*Swanson et al., 1979* *Sekizawa and Shibamoto, 1982*
		S. typhimurium TA100	- -	*Swanson et al., 1979* *Sekizawa and Shibamoto, 1982*	- -	*Swanson et al., 1979* *Sekizawa and Shibamoto, 1982*
		S. typhimurium TA 98	- -	*Swanson et al., 1979* *Sekizawa and Shibamoto, 1982*	- -	*Swanson et al., 1979* *Sekizawa and Shibamoto, 1982*
		S. typhimurium TA 1537	-	*Sekizawa and Shibamoto, 1982*	-	*Sekizawa and Shibamoto, 1982*
		S. typhimurium TA 1538	-	*Sekizawa and Shibamoto, 1982*	-	*Sekizawa and Shibamoto, 1982*
	rec-assay	*Bacillus subtilis* DNA repair test	+	*Sekizawa and Shibamoto, 1982*	N	N
	reversion test	*Escherichia coli* WP2 uvrA	-	*Sekizawa and Shibamoto, 1982*	-	*Sekizawa and Shibamoto, 1982*
	UDS	Rat hepatocytes	-	*Howes et al.1990*	N	N
	CA	CHO SHE	~ +	*Galloway et al., 1987* *Hikiba et al., 2005*	+	*Galloway et al.1987*
1'-hydroxy	Ames test	*S. typhimurium* TA 1535	-	*Swanson et al., 1979*	-	*Swanson et al., 1979*
		S. typhimurium TA100	-	*Swanson et al., 1979*	-	*Swanson et al., 1979*
		S. typhimurium TA 98	-	*Swanson et al., 1979*	-	*Swanson et al., 1979*
2',3'-oxides	Ames test	*S. typhimurium* TA 1535	+	*Swanson et al., 1979*	N	N
		S. typhimurium TA100	-	*Swanson et al., 1979*	-	*Swanson et al., 1979*
		S. typhimurium TA 98	-	*Swanson et al., 1979*	-	*Swanson et al., 1979*
elemicin	UDS	Rat hepatocytes	+	*Hasheminejad and Caldwell, 1994*	N	N
myristicin	UDS	Rat hepatocytes	-	*Hasheminejad and Caldwell, 1994*	N	N

-S9, without metabolic activation; +S9, with metabolic activation; -, negative; +, positive; ~, equivocal (slightly positive); N, No assay performed.

ESTRAGOLE. Controversial results are reported for the mutagenicity of estragole (De Vincenzi et al., 2000). Estragole was tested for mutagenicity in the Ames test with *Salmonella typhimurium* for strains TA98, TA100, TA1535, TA1537 or TA1538, and all gave negative results, with or without S9 mix (Bristol, 2011; Sekizawa and Shibamoto, 1982; Smith et al., 2002), except for strain TA100 where in one of the studies performed estragole was slightly positive without S9 and positive with S9 (Swanson et al., 1979). In another study (To et al., 1982), a significant increase in the revertants per plate was reported for strain TA1538 in the presence of S9 and PAPS (3´ phosphoadenosine 5´-phosphosulfate) cofactors. The authors proposed that the mutagenic response was related to the formation of the sulfate ester of an active metabolite. But, in all the other strains tested by this author (TA98, TA100, TA1535, TA1537), estragole was not mutagenic in assays using PAPS (Smith et al., 2002; To et al., 1982). Estragole was also negative in the *Bacillus subtilis* DNA –repair test (Rec assay) without S9 (4 mg/disk) and in the *Escherichia coli* WP2 *uur*A reversion test with or without S9 (30 to 300µg/plate) (Sekizawa and Shibamoto, 1982).

Estragole metabolites were also studied for mutagenicity. 1'-hydroxyestragole and estragole-2'-3'-epoxide gave positive results in most of the strains studied (Miller et al., 1983; Swanson et al., 1979) except in strain TA98 (Swanson et al., 1979). The electrophilic 2',3'-oxides of estragole and 1'-hydroxyestragole showed dose dependent mutagenic activities for strain TA1535 (Swanson et al., 1979). Estragole metabolites, estragole-2',3'-oxide and 1'-hydroxyestragole-2',3'-oxide showed a dose dependent mutagenic activity for *strains* TA1535 and TA100 without addition of a metabolic activation system (Miller et al., 1983). But in a different study no evidence of mutagenicity was reported for 1´-hydroxyestragole in strain TA100 (Drinkwater et al., 1976).

Another mutagenicity study was performed using the test of Gpt mutant frequency in the liver of male F344 gpt delta rats, estragole was administrated at doses of 22, 66, 200 or 600 mg/kg bw for 4 weeks. Gpt mutant frequency in the liver was increased in a dose-dependent manner, with significance at 200 and 600 mg/kg bw (Suzuki et al., 2012).

Overall, the alkoxyallylbenzene derivatives do not appear to be mutagenic in *S. typhimurium*, being mutagenic in the Gpt mutant frequency assay but only for the higher doses tested.

Regarding genotoxicity, estragole was studied using the UDS assay. UDS was measured using freshly isolated rat hepatocytes in primary culture and estragole induced UDS in a dose-

dependent manner (Chan and Caldwell, 1992; Howes et al., 1990; Muller et al., 1994; Nesslany et al., 2010).

Briefly, in the study by Muller, hepatocytes isolated 4 or 12 h after Wistar male rats received a 500, 1000 or 2000 mg/kg bw doses of estragole which led to a clearly elevated DNA repair for rats treated orally with doses up to 2000 mg/kg bw dose (Muller et al., 1994). In a more recent study male Fischer rats were treated by gavage with a solution of estragole in corn oil and hepatocytes were isolated at 2–4 h or 12–16 h after treatment. The doses administered were 250, 800 and 2000 mg/kg bw and UDS was induced only for two higher doses tested (Nesslany et al., 2010). The doses used in both assays are by far the dose expected to occur in daily basis for human exposure of 0.07 mg/kg bw (EU-SCF, 2001a).

Chromosomal aberrations (CAs) were analysed in V79 cells after exposure to estragole at concentrations of 10^{-3}-10^{-5} M, with and without metabolic activation or in primary rat hepatocytes. Estragole was negative (Muller et al., 1994).

The micronucleus assay in polychromatic erythrocytes, *in vivo*, was negative after treatment of rats with 1000 mg/kg bw and at two lower doses of 500 and 250 mg/kg bw of estragole in solution in corn oil, given in two successive administrations at 24-hour intervals by gavage (Nesslany et al., 2010). No significant increases in the frequencies of micronucleated normochromatic erythrocytes were also observed in peripheral blood samples from male and female mice F344/N rats and B6C3F1 in a 3-month study (Bristol, 2011).

EUGENOL was tested for mutagenic activity in *S. typhimurium strains* TA1535, TA1537, TA1538, TA100 and TA98. No mutagenicity was detected for eugenol with or without the addition of an external source of metabolic activation enzymes (Sekizawa and Shibamoto, 1982; Swanson et al., 1979). Eugenol was positive for mutagenicity in the *Bacillus subtilis* DNA –repair test (Rec assay) without S9 (0,4 mg/disk) and negative in the *Escherichia coli* WP2 *uur*A reversion test with or without S9, at concentrations ranging from 60 to 600 µg/plate (Sekizawa and Shibamoto, 1982). Regarding eugenol metabolites the 2',3'-oxide of eugenol demonstrated to have a dose dependent mutagenic activity for strain TA1535 but not for strain TA98 (Swanson et al., 1979).

Eugenol was also tested with the UDS assay in freshly isolated rat hepatocytes and in female $B6C3F_1$ mice and was negative (Burkey et al., 2000; Howes et al., 1990). Although the negative results using the UDS assay and mutagenicity assays, eugenol was positive in the chromosomal aberrations assay, exhibiting clastogenic activity in SHE cells (195 to 650 µM) that was enhanced in the presence of exogenous metabolic activation (Hikiba et al., 2005). It also gave positive results with S9 activation in several other publications (EFSA, 2009). Assays for sister chromatid exchange in mammalian cells conducted with eugenol gave equivocal results but in human peripheral lymphocytes, no sister chromatid exchange was induced at concentrations less than 500 µM (EFSA, 2009; Jansson et al., 1986).

MYRISTICIN There are few or none available studies regarding myristicin genotoxicity or mutagenicity. The ability of myristicin to induce UDS in rat hepatocytes was addressed in male Fischer 344 rats, for doses higher than 0.5 mM and the results were negative (Hasheminejad and Caldwell, 1994).

ELEMICIN is genotoxic in the UDS assay. Elemicin was studied in hepatocytes derived from male Fischer 344 rats, inducing UDS in a dose-dependent manner, with a maximum response at 0.5 mM (Hasheminejad and Caldwell, 1994).

ADDUCTS

Most chemical carcinogens have to undergo metabolic conversion to strongly electrophilic derivatives, and then interact covalently with nucleophilic sites in cellular macromolecules (DNA, RNA, proteins). Of such reactions, the most probable to occur, are those involving DNA, and DNA adducts formation seems to be a critical event in the initiation of carcinogenesis (Miller and Miller, 1983; Phillips et al., 1981; Randerath et al., 1993).

ESTRAGOLE metabolite 1´-hydroxyestragole, and the corresponding sulfate ester of the 1´-hydroxy metabolites were shown to form DNA adducts *in vivo* and *in vitro* (Smith et al., 2002).

Phillips et al. characterized the DNA adducts formed by 1´-hydroxyestragole. The adducts were isolated from the liver of adult female CD-1 or preweanling B6C3F1, after i.p. injections of 12 µmol/mouse (58mg/Kg) or 0.5 µmol/mouse (14mg/Kg), respectively (Phillips et al., 1981; Phillips et al., 1984). The two major adducts formed were N^2-(estragol-1´-yl)deoxyguanosine and N^2-(trans-isoestragol-3´-yl)deoxyguanosine and other minor nucleoside adducts were found as for N^2-(cis-isoestragol-3´-yl)deoxyguanosine and N^2-(trans-isoestragol-3´-yl)deoxyadenosine. All four adducts found had covalent attachment of the carcinogen moiety to the exocyclic amino group of a purine base (Phillips et al., 1981). Most of the N^2-guanine adducts of 1´-hydroxyestragole were rapidly removed from mouse liver DNA. A significant fraction of each adduct persists, up to at least 20 days after treatment. It may be that some N^2-guanine adducts are repaired more readily than others (Phillips et al., 1981). Minor reaction with N^6 of adenine in mouse-liver DNA was also demonstrated (Randerath et al., 1984).

The capability of alkenylbenzenes to form DNA adducts in the liver of adult female CD-1 mice was also studied by Randerath. Livers were isolated 24 h after i.p. administration of 2 or 10 mg/mouse (100 or 500 mg/kg bw) (Smith et al., 2002) and then analysed with a ^{32}P-post-labelling. Estragole exhibited one of the strongest binding to mouse-liver DNA compared with myristicin and elemicin (Randerath et al., 1984).

Estragole also induced DNA adducts in the liver of wild and reporter gene-carrying F344 rats after gavage administration of 600 mg/kg bw of estragole. Animals were sacrificed at weeks 4, 8 and 16 and DNA adducts were measured by LC-MS/MS in the livers. Estragole-specific DNA adducts were consistently detected, particularly at week 4 (Suzuki et al., 2012).

Allylic epoxides derived from estragole 2´,3´-oxides are also capable of forming covalent adducts with all four deoxyribonucleotides *in vitro* and also occur readily *in vivo* (Guenthner and Luo, 2001). The epoxides formed are sufficiently reactive to easily form covalent bonds with DNA bases but although these metabolites are formed *in vivo* they are also rapidly metabolized to less toxic dihydrodiols or glutathione conjugates (Guenthner and Luo, 2001) by a rapid detoxification

by epoxide hydrolases and glutathione S-transferases (GSTs) (Chen et al., 2011; Guenthner and Luo, 2001). Therefore, the adducts of these 2',3'-oxides were not found in high concentrations in mouse liver following administration of estragole (Chen et al., 2011; Guenthner and Luo, 2001). Comparison of the relative kinetics of epoxide metabolism and epoxide formation also suggests that a wide margin of protection from DNA covalent adduct formation exists in the rat liver and the general rate of epoxide hydrolysis is much greater in human liver than in rat liver (Guenthner and Luo, 2001).

Thus the formation of DNA adducts has been demonstrated and the major hepatic DNA adduct has been characterized as N2 of guanine (De Vincenzi et al., 2000).

EUGENOL. Regarding the assessment of the possible binding of eugenol to liver DNA, Randerath et al. (Randerath et al., 1984) analysed adduct formation in adult female CD-1 mice. After 24 h of an i.p. administration of eugenol (2 or 10 mg/mouse – single dose) adducts were analysed using ^{32}P-post-labelling and eugenol had no biding activity (Randerath et al., 1984).

When analyzing the possibility of anticarcinogenic properties for eugenol, *k-lacZ-transgenic* male mice were fed with a diet containing 0.4% (w/w) eugenol for 58 days. On day 10, half of the mice received an i.p. dose of 100 mg/kg b.w of benzo[a]pyrene. DNA adducts formation of the liver DNA were analyzed by ^{32}P-postlabelling and eugenol had no protective effect. However, one spot indicative of a eugenol-associated DNA adduct was detected which may suggest a possible binding activity for eugenol *in vivo* (Rompelberg et al., 1996b).

MYRISTICIN was capable of inducing DNA adducts *in vitro* in a dose dependent manner, in human hepatoma cells (HepG2). The concentrations used range from 50 to 450 µM and DNA adducts were analyzed by ^{32}P-postlabeling (Zhou et al., 2007).

The capability of myristicin to form DNA adducts in the liver was studied in adult female CD-1 mice, after i.p. administration (2 or 10 mg/mouse – single dose) with ^{32}P-post-labelling assay. Myristicin bound to mouse-liver DNA at 3-200× lower levels compared to the alkenylbenzenes safrole, estragole or methyleugenol (Randerath et al., 1984; Randerath et al., 1985).

Thus, these studies suggest that myristicin can have a binding capability. In another study, cola drinks were tested for adducts, and the authors found adducts identical to those formed by myristicin. Cola drinks were given in place of drinking water to mice for up to 8 weeks, and mice developed significant levels of covalent liver DNA adducts. The adducts induced by cola drinks were chromatographically identical to those detected in mice treated with extracts of nutmeg or mace, or with myristicin (a major compound constituent of nutmeg). Liver DNA adducts were also detected in the fetal liver when pregnant mice were intubated with myristicin (Randerath et al., 1993). Overall, DNA adducts induced by cola drinks, nutmeg and mace, in each case, coincided on the chromatograms, with the corresponding myristicin-DNA adducts (Randerath et al., 1993).

ELEMICIN In the study by Randerath (Randerath et al., 1984), with adult female CD-1 mice, in the same conditions as for myristicin, elemicin also bound to mouse liver DNA 4 to 200 times less than safrole, estragole and methyleugenol (Randerath et al., 1984).

CARCINOGENICITY

Regarding the carcinogenicity of alkenylbenzenes, myristicin, elemicin and eugenol have little or no carcinogenic activity compared to the hepatocarcinogenic activities of safrole and estragole in male mice (Miller et al., 1983), although regarding the first three alkenylbenzenes, in what concerns long term studies, fewer studies are available. These different activities found between alkenylbenzenes seems to depend on the number and positions of methoxy and methylenedioxy substituents on the benzene ring (Miller et al., 1983; Silva et al., 2000).

Safrole was one of the first alkenylbenzenes studied regarding carcinogenicity. Safrole is capable of inducing liver tumours in mice and rats (Chen et al., 2011) and the carcinogenicity of safrole metabolites 1'-hydroxysafrole, safrole-2',3'-oxide and 1'-hydroxysafrole-2',3'-oxide was

clearly demonstrated in rodents (Drinkwater et al., 1976). The studies performed with safrole, demonstrated a relation between safrole-adduct formation and carcinogenicity. In mice studies, safrole and cola beverages induced safrole-DNA adducts in the liver, the same adducts were found in human peripheral blood, tissue samples from patients with esophageal and liver cancer. In liver cancer patients who had a history of betel quid chewing (major source of safrole), safrole-DNA adduct were identified, as for patients who consume large amounts of nutmeg (Chen et al., 2011; Randerath et al., 1993).

ESTRAGOLE. Regarding carcinogenicity studies, estragole was capable of inducing tumors in mice (Bristol, 2011; EU-SCF, 2001a, b, 2002).

Estragole, was first assessed for carcinogenicity in 1976 (Drinkwater et al., 1976), in this particular study estragole and its 1'-hydroxy metabolite caused significant increases in the incidences of hepatocellular carcinomas in male CD-1 mice that received estragole by s.c. injection at 1-22 days of age. The doses administrated were of 4.4 and 5.2 µmoles in a 15 months assay (Drinkwater et al., 1976).

Miller at al. also tested hepatocarcinogenicity of estragole and its proximate carcinogenic metabolite 1´-hydroxyestragole in preweanling (administrated i.p. at total doses of up to 9.45 µmol/mouse to male CD-1 or B6C3F_1 mice) and adult mice. Hepatic tumours were formed in male CD-1 mice during the preweaning period and on administration for 12 months in the diet of female CD-1 mice (Miller et al., 1983). The 2´,3´-oxides of estragole were also tested but had little or no activity, as for the induction of lung adenomas in female A/J mice or for the induction of tumours on repetitive injections s.c. in male Fischer rats, providing little evidence for their importance as carcinogenic metabolites in vivo (Miller et al., 1983).

Carcinogenicity studies in rat liver, particularly in F344 male rats, wild and reporter gene-carrying, were performed exposing rats to estragole by gavage administration of 600 mg/kg bw, for 4 to 16 weeks and GST-P-positive liver cell foci were measured (rat liver preneoplastic lesions

detectable in the medium-term assay). Estragole increased GST-P-positive liver cell foci which may suggest that estragole may also exert hepatocarcinogenicity in rats (Suzuki et al., 2012).

Overall, estragole had hepatocarcinogenic activity similar to that of safrole in mice given injections of the compound prior to weaning (Drinkwater et al., 1976). Nevertheless, dose levels used in carcinogenicity studies have been very much higher than the estimated human daily intake (0.07 mg/Kg bw). Moreover the percentage of an administrated dose of estragole eliminated as 1'-hydroxyestragole glucuronide in human urine is much lower than that found with even the lowest doses examined in rats (Anthony et al., 1987; Sangster et al., 1987). The excretion of 1´-hydroxyestragole glucuronide in human urine amounts to only 0.3% of the administrated dose, or 0.02 nmol/Kg/24h, a value far lower than that obtained in rodents even at the lowest doses examined. It is thus possible that the rodent carcinogenicity tests may overestimate the risk accruing from estragole at normal levels of usage (Anthony et al., 1987; Chen et al., 2011; Smith et al., 2002).

EUGENOL. Regarding carcinogenicity studies, eugenol is considered a non-carcinogenic compound. Hepatocarcinogenicity was studied in rats and mice by the National Toxicology Program for a long-period of two years (NTP, 1983) and no carcinogenicity was found. Only one equivocal result was reported by the NTP for mice in which eugenol caused increased incidences of both carcinomas and adenomas of the liver in male mice only for those treated with the lower dose of eugenol and eugenol was also associated with an increase in the combined incidences of hepatocellular carcinomas or adenomas in female mice (NTP, 1983). Despite this positive result other authors did not considere it relevant (Rompelberg et al., 1993).

Miller at al. also tested eugenol hepatocarcinogenicity in male CD-1 mice at similar doses but for a shorter period of 12 months. Eugenol 2´,3´-oxides metabolites had little or no activity (Miller et al., 1983). However, the 2´,3´-oxides when administrated topically to female CD-1 mice at relatively high doses (45 µmol/week, during 6 weeks), initiated benign skin tumours that could be promoted with croton oil (Miller et al., 1983).

MYRISTICIN was tested for carcinogenicity in adult female CD-1 and preweanling mice, and no carcinogenic activity was demonstrated (Miller et al., 1983). Hepatocarcinogenicity was also tested in B6C3F1 male mice on administration of myristicin prior to weaning (total dose of 4.75µmol/mouse) and no detectable activity was seen for the initiation of hepatic tumours (Miller et al., 1983).

While safrole is an established hepatocarcinogen in rats and mice, myristicin, despite their structural similarity, is not. But both myristicin and safrole are capable of inducing transplacental liver DNA damage, which is of concern because the rapid division of fetal cells facilitates expression of such lesions, contributing to mutagenesis and potentially carcinogenesis (Randerath et al., 1993).

Moreover, toxicogenomics and machine learning models were used for the prediction of hepatocarcinogenic potential of alkenylbenzenes and myristicin was considered as a potential weak hepatocarcinogenic if studied at a dose level of 2 mmol/kg bw/day for 2 years in male F344 rats. Therefore this models suggest that myristicin could be of higher priority relative to other alkenylbenzenes for evaluation in a long term carcinogenicity assay (Auerbach et al., 2010).

ELEMICIN. For elemicin it is important to address the limited studies available. The few studies performed for carcinogenicity of elemicin regards the study by Miller et al. (Miller et al., 1983). Elemicin and its 1'- hydroxy metabolite was tested at 4.75 µmol/mouse (49.5 mg/kg bw) in male mice prior to weaning ($B6C3F_1$) and had no detectable activity for the initiation of hepatic tumors (Miller et al., 1983).

Moreover, tumor induction in male $B6C3F_1$ mice given *i.p.* injections of 1'-hydroxyelemicin prior to weaning was found to be 30-fold lower, on a molar basis compared to the results found for its structurally related analogue 1'-hydroxyestragole.

These differences in DNA binding activities and subsequent tumour formation between the different alkenylbenzenes might be explained by the fact that increasing the number of methoxy substituents will decrease the electrophilicity of their 1'- sulfoxymetabolites and/or the corresponding carbocations which is in line with the fact that mono- and di-substituted alkenylbenzenes were bound more rapidly to mouse liver DNA and showed higher levels of

hepatocarcinogenic activity compared to structurally related analogues exerting multiple methylenedioxy and/or methoxy groups.

AIM AND OUTLINE

The aim of this thesis was to contribute to the risk assessment of the alkenylbenzenes family. Committees from the European Commission, and other regulatory authorities, recommend restrictions in the use of some alkenylbenzenes until further studies are carried out to clarify the uncertainty on their genotoxicity and carcinogenic potential, and the nature and implications that can accrue from exposure to low and prolonged levels of exposure.

Exposure to this group of compounds arises mainly from spices and their essential oils, e.g. fennel, clove, nutmeg and tarragon, from their use as flavourings and in herbal preparations, e.g. teas and medicines. Risk assessment is therefore a priority for this group of compounds.

Therefore, at first our aim was to assess the cytotoxicity and genotoxicity using assays in Hamster cells (V79 and CHO cells), specifically MTT for cytotoxicity and Chromosomal Aberrations and the Sister chromatid exchange assays, for genotoxicity.

Estragole, eugenol and myristicin are grouped in the same family, but present structural differences, and thus, depending on their behavior different assays were executed to assess possible mechanisms of action. To analyze DNA break-inducing capability we used the comet assay (measures essentially SSB), and the gama-H2AX assay (measures DSBs). Depending on the lesion induced further studies were performed such as DNA adduct formation. One of the DNA Damage Response (DDR) mechanisms is apoptosis, thus to study additional consequences of DNA damage we performed several assays to analyse the different events of the intrinsic apoptotic pathway. The effect of exposure on the expression levels of different genes, namely of the DDR pathway were also studied by RT-PCR. A prove of concept study involving epigenetic alterations, e.g. DNA methylation, was also analyzed as a possible mechanism of gene expression alterations induced by these alkenylbenzenes.

Most studies in toxicology have analyzed the exposure of high doses of xenobiotics for a short period of time, typically during 1-2 cycles. This methodology does not reveal the biological

effects, namely epigenetic effects, after prolonged exposure to low doses, more in line with chronic exposure to dietary components. Keeping this in line we analysed methylation alterations in promoter regions of several genes after prolonged exposure (15 days) to a low dose of alkenylbenzenes.

Overall, the studies outlined enable us to gather additional information on the genotoxic mechanisms of the alkenylbenzenes family, providing further material for risk assessment of this group of compounds.

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2. Chapter

GENOTOXICITY AND ENDOREDUPLICATION INDUCING ACTIVITY OF THE FOOD FLAVOURING EUGENOL

This chapter was adapted from:

Genotoxicity and endoreduplication inducing activity of the food flavouring eugenol

Maralhas A., Monteiro A., Martins C., Kranendonk M., Laires A., Rueff J., and Rodrigues A.S. Mutagenesis (2006) 21, 199–204.

ABSTRACT

Eugenol, a compound extracted from clove oil and marjoram, is widely used as a food flavouring substance and is present in spices such as basil, cinnamon and nutmeg. It is also used in dentistry as an antiseptic and analgesic. A structural similarity with the class IIB IARC carcinogen safrole raises questions on its putative carcinogenicity. We evaluated the genotoxicity of eugenol in V79 cells using SCE and CAs assays, with and without rat liver biotransformation (S9). Eugenol induced CAs, with significant increases (6.5% aberrant cells) at 1000 µM in the presence of S9 mix in a dose-dependent manner, with a high frequency of chromatid exchanges. In particular, an increase of endoreduplicated cells was observed, from 0% at control levels to 2.3 and 5% at 2000 µM, without and with S9, respectively. Since endoreduplication has been linked to inhibition of topoisomerase II, the topoisomerase II inhibitor ICRF-193 was used as a control inducer of endoreduplication (0.1–0.5 µM), increasing the number of endoreduplicated cells from 0% (control) to 3.5% and 3.1 % (at 0.5 µM) without and with S9 mix, respectively. S9 did not influence endoreduplication by ICRF-193. Both eugenol and ICRF-193 were also assayed for inhibition of topoisomerase II, and both showed a dose-dependent inhibitory effect, with ICRF-193 being a more potent inhibitor at lower doses. Our results confirm that eugenol is genotoxic and raises the possibility of it having topoisomerase II inhibiting activity.

2.1 INTRODUCTION

The large-scale use of certain food flavourings requires accumulation of toxicological data on these substances, particularly in cases where structural similarities with other known substances showing genotoxic or carcinogenic properties indicate that restrictions on human consumption or exposure should be implemented (Bolt et al., 2004; Marques et al., 2002; Schrankel, 2004). This is the case of the flavouring substance eugenol, structurally similar to safrole, a class 2B IARC

carcinogen. In all these cases human exposure is widespread through consumption of food and beverages, raising the possibility of adverse effects in human populations (Jeffrey and Williams, 2005). Eugenol (1-allyl-3-methoxy-4-hydroxybenzene; CAS No.97-53-0) is present in a variety of essential oils, such as clove oil (85–95% eugenol), marjoram essential oil (10%), ground cinnamon (0.02%), ground cloves (1%), clove oleoresin (60–90%) and in cinnamon leaf oil (70–90%) (NTP, 1983). Eugenol is also used widely as an analgesic and antiseptic in clinical dentistry. The yearly worldwide production of eugenol is estimated at ~22,000 Kg (Smith et al., 2001), and it is used mostly by the food, perfume and pesticide industry (IARC, 1985).

The average maximum used levels in food products, such as beverages, ice cream, baked goods, gelatins and puddings, and chewing gums, range from 1.4 to 500 p.p.m. (NTP, 1983). Although the ADI was revised by the International Programme on Chemical Safety in 1982 to 0–2.5 mg/kg bw (JECFA, 1982), estimates of average human consumption of eugenol vary from 7 to 76 μg/day (Smith et al., 2001). No further evaluation has been performed by major health risk organizations. The Scientific Committee on Food of the European Commission could not establish a safe exposure limit for eugenol. This is in contrast to other flavourings for which an ADI has been calculated.

Although eugenol's toxicity has been studied in laboratory animals (NTP, 1983), little or no information is available on human exposure and subsequent possible adverse health outcomes. Both toxicological and human exposure data are needed to make accurate risk evaluations. From a chemical point of view, eugenol is a alkenylbenzene derivative, similar to estragole, methyleugenol and safrole. Owing to its structural similarity to the carcinogen safrole, there is concern about its carcinogenic and mutagenic properties (Rietjens et al., 2005). The carcinogenicity of eugenol was evaluated by the US National Toxicology Program (NTP) with negative outcomes for F344 rats of either sex, whereas results for male and female B6C3F1 mice were considered equivocal (NTP, 1983). As for its genotoxicity, results were negative in various *Salmonella typhimurium* strains, either with or without metabolic activation. Results in other test systems showed genotoxic activity in Chinese hamster ovary cells, using CAs as endpoints (NTP, 1982). *Hikiba et al.* (Hikiba et al., 2005) evaluated the genotoxicity of eugenol together with other chemicals used in dental practice and observed an induction of CAs in Syrian hamster embryo

cells. The presence of exogenous metabolic activation enhanced the genotoxicity of eugenol. The Drosophila wing somatic mutation and recombination test (SMART) was used to evaluate the recombinogenic activity of eugenol, with positive results in a variant having increased cytochrome P450 activity (Munerato et al., 2005), once again emphasizing the need for metabolic activation for increased genotoxicity.

Accumulated evidence suggests that the biotransformation of eugenol should be similar to the biotransformation of other alkenylbenzene derivatives such as estragole and methyleugenol (Burkey et al., 2000; Guenthner and Luo, 2001; NTP, 2000; Rietjens et al., 2005; Smith et al., 2002; Thompson et al., 1989; Thompson et al., 1998). Thus, eugenol would undergo three metabolic reactions, hydroxylation, epoxidation and *O*-demethylation. The reactive metabolite, 2,3-epoxyeugenol, and 1´- hydroxyeugenol, after originating the quinone methide, can react with DNA, forming adducts (Bodell et al., 1998; Burkey et al., 2000; Guenthner and Luo, 2001; Sakano et al., 2004; Smith et al., 2002) that can contribute to the genotoxic activity of eugenol.

Following our interest on the mechanisms of genotoxicity of food compounds (Alves et al., 2000; Duarte et al., 2000; Marques et al., 2002; Rueff et al., 1992; Silva et al., 2000), we report here results on the genotoxicity of eugenol in V79 Chinese hamster cells, with and without exogenous biotransformation, using SCE and CAs as endpoints. The observation that eugenol induced endoreduplication (ER) in these cells led us to evaluate this endpoint, both with and without exogenous biotransformation. Since ER has been linked to topoisomerase II (topo II) inhibition (Cortes and Pastor, 2003), the bis-2,6-dioxopiperazine derivative ICRF-193 was used both as a reference inhibitor of topo II and an inducer of ER (Hajji et al., 2003).

2.2 MATERIAL AND METHODS

2.2.1 Chemicals and Reagents

Eugenol (99%), mitomycin C (MMC), newborn calf serum and Ham's F-10 medium, penicillin and streptomycin, 5-bromo-2'-deoxiuridine (BrdU), bisbenzamide (2'-[4-hidroxifenil]-5-[4-metil-1-piperazinil]-2,5'-bi-1H-benzimidazol), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium

bromide (MTT) and cyclophosphamide (CP) were purchased from Sigma (St Louis, MO). Dimethylsulfoxide (DMSO), methanol, acetic acid, potassium chloride and Giemsa dye were obtained from Merck (Darmstadt, Germany). Colchicine was purchased from Fluka (Buchs, Switzerland). Trypsin was obtained from Difco Laboratories (Detroit, Mich). ICRF-193 (meso-2,3-bis(3,5-dioxopiperazine-1-yl)butane was purchased from Zenyaku Kogyo Co., Japan. Aroclor 1354 induced rat liver S9 fractions were purchased from Trinova Biochem GmbH (Giessen, Germany).

2.2.2 Cell Culture

The wild-type V79 Chinese hamster cells used in this study were kindly provided by Prof. H.R. Glatt (German Institute of Human Nutrition, Berlin, Germany). The cells were routinely maintained in 175-cm^2 culture flasks (Sarstedt, AG&CO, Nümbrecht, Germany) using Ham's F-10 medium, supplemented with 1% antibiotic solution (penicillin-streptomycin) and 10% newborn calf serum and incubated at 37ºC in an atmosphere containing 5% carbon dioxide.

2.2.3 MTT viability assay

Approximately 5×10^3 cells were cultured in 200 µl of culture medium per well in 96-well plates and incubated at 37 ºC under a 5% CO_2 atmosphere. The cells were grown for twenty four hours and then exposed to different concentrations of eugenol, ranging from 10 to 5000 µM, for 3 h and 24 h periods. The cells were washed with culture medium and MTT (dissolved in culture medium) was added to each well at a concentration of 0.5 mg/ml. The cells were grown for a further period of 3 h and then carefully washed with phosphate buffered saline (PBS). At the end of the incubation period, the media was discarded and DMSO (200 µl) was added to each well to dissolve the formazan crystals. Absorbance was read at 595 nm in a Zenyth 3100 microplate reader. At least two independent experiments were performed.

2.2.4 Sister chromatid exchange assay (SCE)

V79 cells at approximately 5 x 10^5 cells/flask, that add been cultured for twenty four hours in 25-cm^2 culture flasks, were exposed to different final concentrations of eugenol ranging from 100 to 2500 µM, with and without an exogenous metabolic activation system, S9 mix (10% v/v),

prepared as described previously (Maron and Ames, 1983). Two hours later the cells were washed with fresh culture medium and BrdU was added at a final concentration of 6 µM. After 25 h, colchicine was added at a final concentration of 600 ng/ml. The cells were then incubated for a further 3 h period and then harvested by trypsinization. Following a 3 min hypotonic treatment with KCl (0.56%, w/v) at 37ºC, the cells were fixed with methanol/acetic acid (3:1). As positive controls we used MMC (750 nM) (data not shown) and cyclophosphamide (20 µM), for tests without and with metabolic activation, respectively.

Differential staining of BrdU-substituted sister-chromatids was performed according to the fluorescence-plus-Giemsa (FPG) method (Perry and Wolff, 1974). Briefly, the slides were stained for 12 min with the fluorescent dye Hoescht 33258 (10 µg/ml) in 2% KCl (w/v), exposed to UV (254 nm) for approximately 9 min, and then stained with 4% Giemsa (v/v) in 10 mM phosphate buffer (pH 6.8) for 10 min.

SCE per cell were scored in 30 s-metaphases for each dose-level in each experiment. Two independent experiments were performed, with and without S9 mix.

2.2.5 Chromosome Aberrations Assay (CAs)

Twenty-four hour cultures (~5×10^5 cells/flask) growing as monolayers in 25-cm^2 tissue culture flasks were treated with different doses of eugenol (100 – 3000 µM) or ICRF-193 (0.1 – 0.5 µM), both dissolved in DMSO, for 3 h. When testing in the presence of a biotransformation system, 500 µl of 10% (v/v) S9 mix, prepared as described previously (Maron and Ames, 1983), was added to the flasks. In all the experiments, the cells were then washed and incubated with fresh medium for an additional period of 13 h. Colchicine was then added at a final concentration of 6.5 mM. Cells were grown for a further 3 h and then harvested by trypsinization.

After 3 min hypotonic treatment with KCl 75 mM at 37ºC, cells were fixed with ice-cold methanol/acetic acid (3:1) and slides were prepared and stained with Giemsa at 4% (v/v) in 10 mM phosphate buffer (pH 6.8), for 10 min. Two to three independent experiments were carried out for each dose and 100 metaphases were scored. Control cells included DMSO as negative control, which did not exceed 0.5% (v/v) of the culture medium, and MMC as positive control (0.36 µM) in the absence of S9 mix and CP (21.5 µM) in the presence of S9 mix. The mitotic indices

were estimated by counting at least 1000 cells. The statistical analysis for the comparison of each test dose of eugenol to the DMSO control was performed using the two-tailed Student's *t*-test.

2.2.6 Endoreduplication assay

Slides prepared for the CAs assay were analysed for the presence of endoreduplicated metaphases by counting 2000 metaphases and scoring ERs.

2.2.7 Topoisomerase II activity Assay

Topoisomerase II (topo II) activity was assayed using a TopoGen (Columbus, OH) assay kit based on the decatenation of supercoiled plasmid. The pRYG is a plasmid containing a single, high affinity topo II cleavage and recognition site. The amount of DNA substrate (pRYG) used in each assay was 0.04 pmol (Nakamura et al., 2002). Immediately before use, test and control compounds were diluted in 1% (v/v) DMSO to maintain DMSO concentration at or below 1% of the final incubation volume. Eugenol was added to the reaction mix at concentrations of 5 – 7500 µM and ICRF-193 was used as positive control at concentrations of 1 – 10 µM. After 60 min incubation at 37ºC, the samples were loaded onto 1% agarose gel and subjected to electrophoresis for 1.30 h at 100 V/cm. Finally the gel was stained (45 min) with 0.5 µg/ml ethidium bromide, destained in distilled water (10 min) and photographed with Fujifilm S500 digital camera.

2.3 RESULTS

2.3.1 Viability assay

A wide range of eugenol concentrations were tested in a 3 and 24 h incubation periods using the MTT viability assay protocol. The survival values obtained are depicted in Figure 2.1. It is clear that eugenol induced a dose-dependent cell death for both times periods. For the 3 h period viability was below 50% at concentrations higher than 2500 µM, with mean values of 36.2% for the concentration of 3000 µM. For the 24 h exposure period eugenol was more cytotoxic, causing

lower survival rates (~ 10%) at concentrations higher than 1500 µM, with mean values of 11.0 % and 10.1 % for the concentrations of 2000 µM and 2500 µM, respectively.

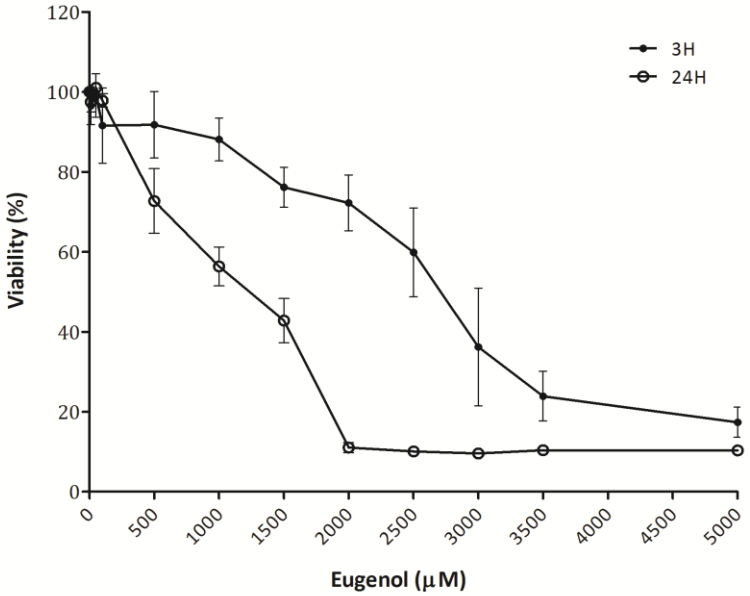

Figure 2.1 MTT viability assay in V79 cells, after a 3 and 24 h of exposure to eugenol. The results are expressed as mean values ± SEM from at least two independent experiments.

2.3.2 Sister chromatid exchange assay (SCE)

Regarding this indicator of genotoxicity, eugenol did not induce significant SCE, either in the presence or absence of S9 mix, in V79 cells as we can see in Figure 2.2 (B). Although there was a slightly increase with S9 mix, it was not significant and it had no dose response effect. Figure 2.2 (A) shows an image with 16 exchanges (7.6 SCE/cell) after exposure to 100 µM of eugenol compared to the negative control, in the first image, with 7 exchanges (5.08 SCE/cell) and with the positive control CP, the third image, with more than 30 exchanges.

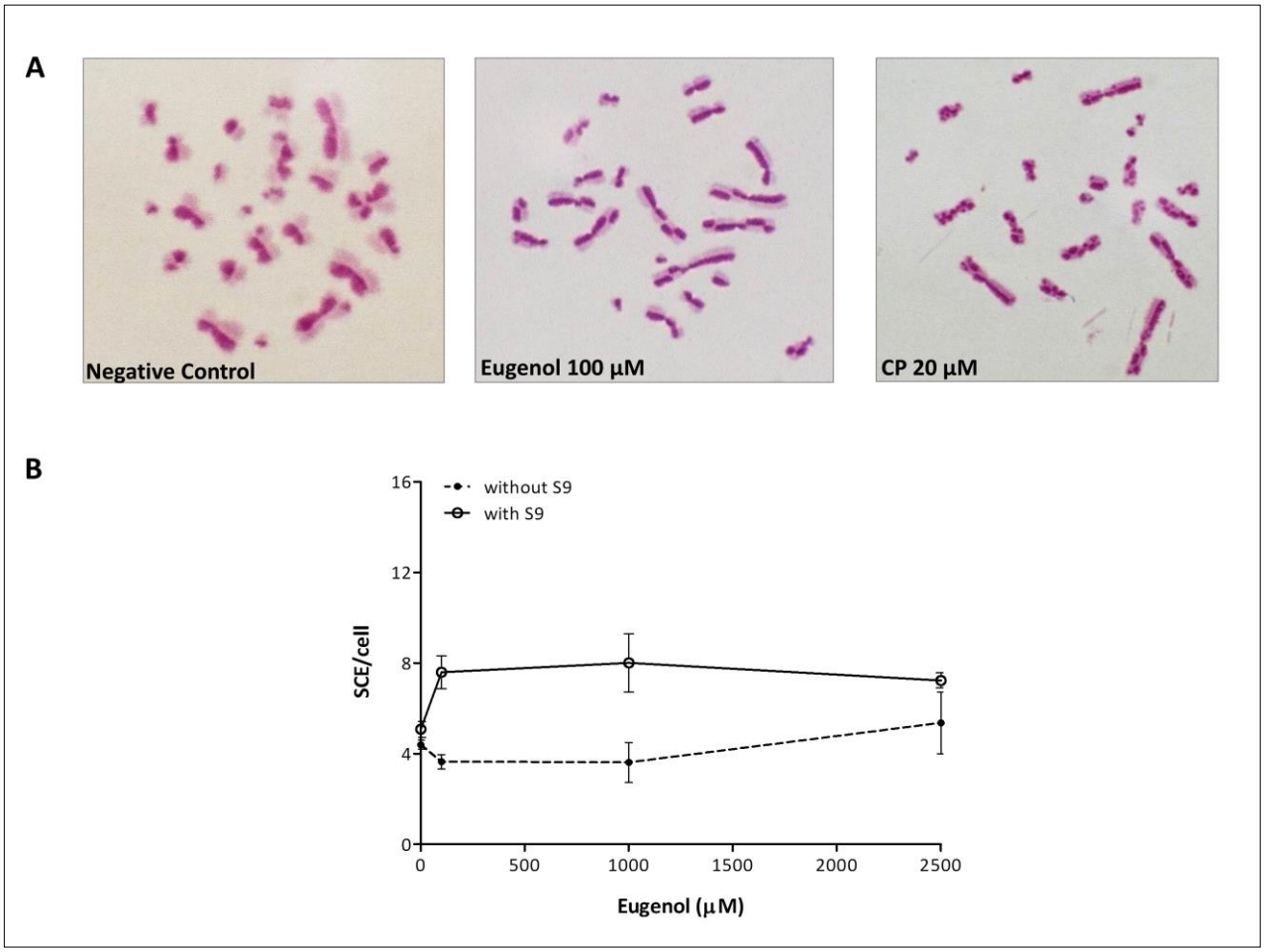

Figure 2.2 Sister chromatid exchanges induced by eugenol in V79 cells. SCE induction was analyzed in the absence and presence of S9 mix after a 2 h exposure period. (A) Images show analysis of SCE induction in the presence of S9 mix. (B) Sister chromatid exchange per cell (SCE/cell) expressed as the average ± SEM from two independent experiments, in the absence and presence of S9 mix.

2.3.3 Chromosome Aberrations

Results of CAs induced by eugenol in V79 cells are shown in Table 2.1 and Figure 2.3. Eugenol induced a dose-dependent increase of aberrant cells excluding gaps (ACEG) in V79 cells up to 3000 µM, in the presence of S9 mix (Figure 2.3 (B)), with significant increases when compared to the control (0.5 % CAEG). Eugenol induced 6.5 % CAEG for the concentration of 1000 µM and 15% for 2000 µM, with particular emphasis on chromatid exchanges, as we can see in the images regarding eugenol from Figure 2.3 (A). In the absence of S9 mix, however, the genotoxicity of eugenol was not as clear, although there was an increase in aberrant cells from 0% (control) to 3.5% aberrant cells at 2500 µM, demonstrating cytotoxicity at higher doses, as indicated by the

mitotic index (Table 2.1) and the MTT assay (Figure 2.1). Multi-aberrant cells are defined as cells containing more than 10 CAs, excluding gaps. These cells are included in the scores concerning aberrant cells (%). The individual CAs present in the multiaberrant cells are not included in the scores containing total aberrations. The increase in CAs in the presence of S9 mix indicates a contribution of biotransformation enzymes to the genotoxicity of eugenol.

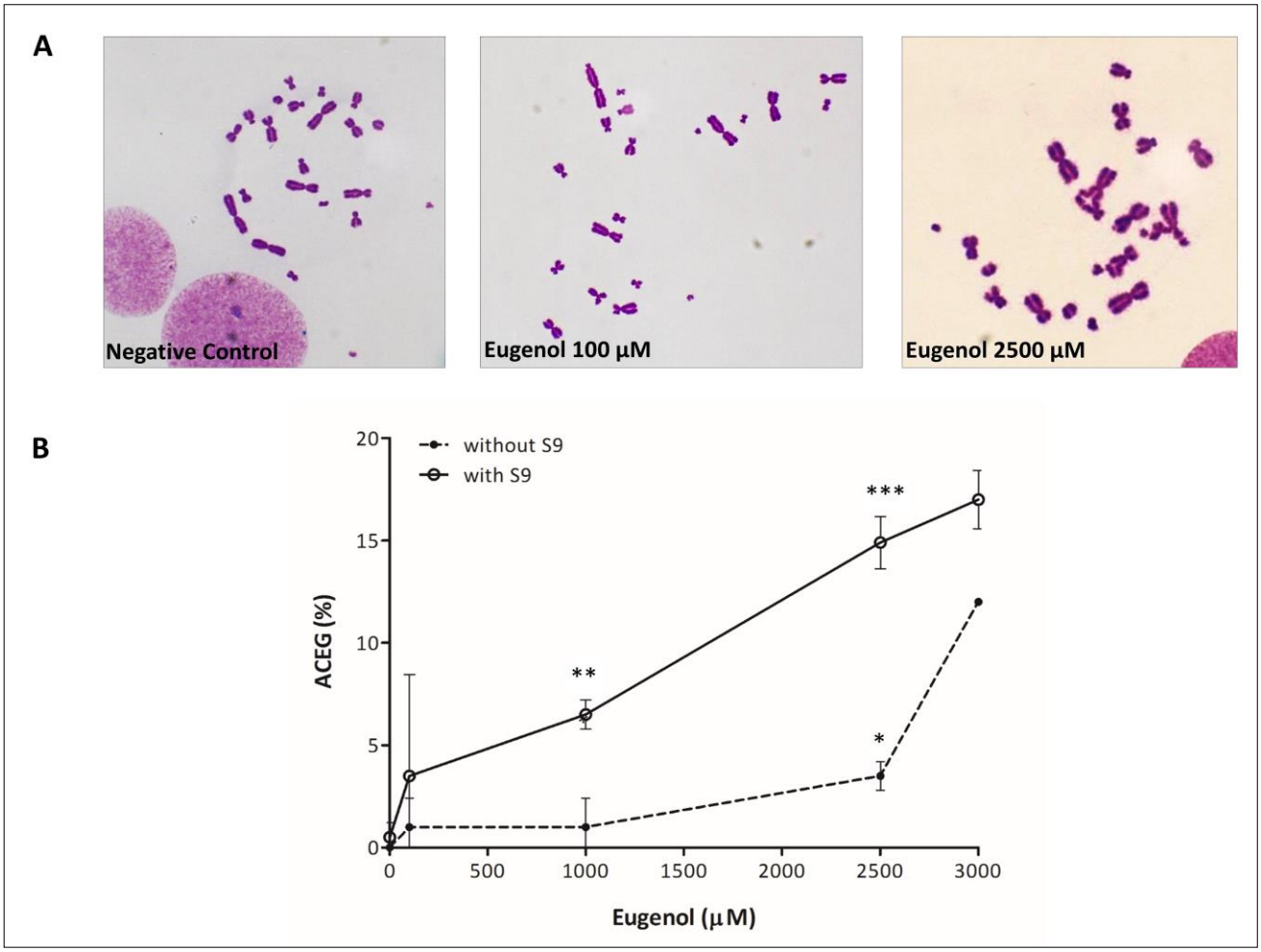

Figure 2.3 Chromosomal aberrations induced by eugenol in V79 cells. (A) Images of CAs induction in V79 cells, in the presence of S9 mix after a 2 h incubation period. (B) Percentage of chromosomal aberrant cells excluding gaps (%ACEG), in the absence and presence of S9 mix after a 3 h incubation period. Results are expressed as the average ± SD from two independent experiments for all the points, except for the 3000 µM concentration without S9. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.005$, Student's *t*-test.

Table 2.1 Induction of CAs by eugenol in V79 cells, in the absence and presence of S9 Mix, after a 3 h incubation period.

Test Compound	Chromosomal Aberrations per 100 cells[a]								% ACEG	CAEG/cell	MI (%)
	S9 Mix	Ctg	Csg	Ctb	Csb	Exch	Dic + Oth	>10			
Eugenol (μM)											
0	-	3.5	0.5	0	0	0	0	0	0 ± 0	0	2.1 ± 0.9
100	-	6.5	0.5	0	0.5	0	0	0.5	1.0 ± 1.4	0.01	1.1 ± 0.1
1000	-	4.0	0	0.5	0	0	0	0.5	1.0 ± 1.4	0.01	1.1 ± 0.1
2500	-	7.0	0.5	1.0	1.0	0	1.0	0.5	3.5 ± 0.7*	0.04	0.6 ± 0.2
3000	-	13.0	0	6.0	0	2.0	0	4.0	12.0	0.12	–
0	+	4.0	0	0.5	0	0	0	0	0.5 ± 0.7	0.01	5.7 ± 4.7
100	+	2.5	0	3.0	0	0	0.5	0	3.5 ± 5.0	0.04	7.9 ± 9.6
1000	+	4.0	0	3.0	0	3.0	0.5	0	6.5 ± 0.7**	0.07	4.0 ± 4.0
2500	+	3.0	0	7.5	0	7.0	0.5	0	15.0 ± 1.4***	0.15	2.3 ± 2.6
3000	+	4.0	1.0	6.0	1.0	2.0	9.0	0	18.0 ± 12.7	0.18	0.4 ± 0.5
MMC (μM)											
0.36	-	11.5	1.0	9.5	0.5	2.5	0.5	0	10.4 ± 4.8	0.13	3.8 ± 1.1
CP (μM)											
21.5	+	3.0	0	3.5	0.5	0.5	0	0	4.5 ± 2.1	0.05	7.8 ± 0.6

[a] Results refer to average values of two independent experiments (100 metaphases analysed per experiment) for all experimental points except for 3000 μM where results are of one experiment without S9 Mix. Ctg: chromatid "gap"; Csg: chromosome "gap"; Ctb: chromatid break; Csb: chromosome break; Exch: triradial, quadriradial and other chromatid exchanges; Dic + Oth: dicentric chromosomes and other aberrations (e.g. rings); >10: multiaberrant cells; % ACEG: percentage of aberrant cells excluding "gaps" (average ± standard deviation); CAEG/cell: chromosomal aberrations excluding "gaps" per cell; MI: mitotic index; MMC: mytomicin C; CP: cyclophosphamide.* $P < 0.05$ (Student's *t*-test) when compared to the control,** $P < 0.01$ (Student's *t*-test) when compared to the control, *** $P < 0.005$ (Student's *t*-test) when compared to the control.

2.3.4 Endoreduplication and Topoisomerase II activity

The observation that eugenol induced ER (Figure 2.4, images (A)) led us to evaluate this endpoint, with and without S9 mix. The results obtained for endoreduplicated cells are shown in Tables 2.2 and 2.3, and Figure 2.4. As can be seen, eugenol induced a dose-dependent increase in the percentage of endoreduplicated cells, in the absence and presence of rat liver biotransformation system S9. An increase in endoreduplicated cells was observed, from 0% at control levels to 2.25 % at 2000 μM (Table 2.2), which increased in the presence of S9 (5.07 % at 2000 μM) (Table 2.3). The increase in ER in the presence of S9 mix once again suggests a contribution of biotransformation enzymes to the ER inducing activity of eugenol. As a positive control inducer of ER, the bisdioxopiperazine ICRF-193, a known topoisomerase II inhibitor, was chosen. ER induced a dose-dependent increase in endoreduplicated cells, as expected (Pastor et al., 2002).

These results are presented in Tables 2.2 and 2.3 and shown in Figure 2.5. ICRF-193 is a more potent inducer of ER than eugenol. The presence of the rat liver S9 did not alter the endoreduplicating inducing activity of ICRF-193.

Since ER has been linked to inhibition of the enzyme topo II, the activity of this enzyme was assayed in the presence of eugenol (Figure 2.6). The results show that eugenol inhibits topo II in a dose-dependent way, albeit at concentrations well above ICRF-193 as we can see in Figure 2.6.

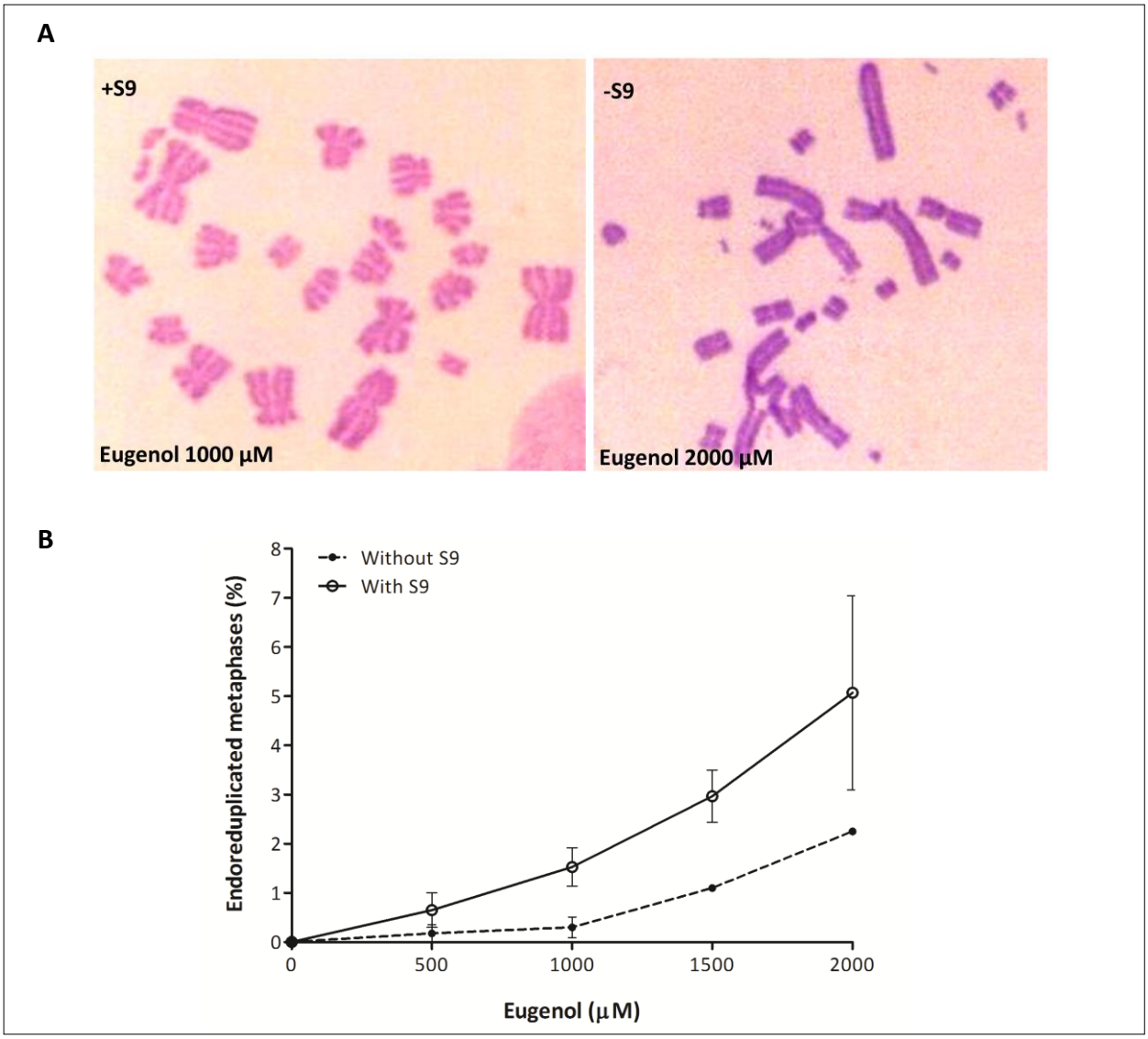

Figure 2.4 Induction of endoreduplication (ER) by eugenol in V79 cells. (A) Images of endoreduplication induction in V79 cells, in the presence and in the absence of S9 mix after a 3 h incubation period. (B) Percentage of endoreduplicated cells scored in 2000 metaphases after a 3 h incubation period with eugenol, in the absence and presence of S9 mix. Results are expressed as the average ± SD from at least two independent experiments.

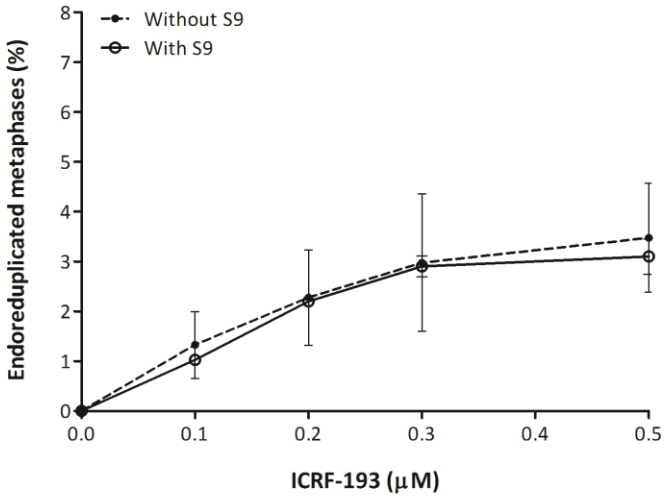

Figure 2.5 Induction of Endoreduplication (ER) by ICRF-193 in V79 cells.Values are shown as the percentage of endoreduplicated cells scored in 2000 metaphases, in the presence and in the absence of S9 mix after a 3 h incubation period. Results are expressed as the average ± SD from two independent experiments.

Table 2.2 Induction of endoreduplication by eugenol and ICRF-193 in V79 cells, in the absence of S9 Mix, after a 3 h incubation period.

Test Compound	S9 Mix	Endoreduplications per 2000 metaphases	% Endoreduplication	MI (%)
Eugenol (μM)				
0	-	0 ± 0	0 ± 0	7.10 ± 0.57
500	-	3.5 ± 3.5	0.18± 0.18	3.95 ± 0.21
1000	-	6.0 ± 4.2	0.30 ± 0.21	3.40 ± 0.99
1500	-	22.0 ± 1.4*	1.10 ± 0.07	3.20 ± 0.99
2000	-	45.0 ± 1.4*	2.25 ± 0.07	2.65 ± 0.35
2500	-	a	a	0.05 ± 0.07
ICRF-193 (μM)				
0	-	0	0 ± 0	6.80 ± 1.22
0.1	-	26.5 ± 0	1.33 ± 0.67	6.00 ± 2.18
0.2	-	45.5 ± 13.4*	2.28 ± 0.95	4.47 ± 1.78
0.3	-	59.5 ± 19.1	2.98 ± 1.38	3.57 ± 1.24
0.5	-	69.5 ± 21.9	3.48 ± 1.10	3.07 ± 1.16

[a] High levels of cytotoxicity were observed at this concentration.*$P < 0.05$ (Student's *t*-test) when compared to the control.

Table 2.3 Induction of endoreduplications by eugenol and ICRF-193 in V79 cells, in the presence of S9 Mix, after a 3 h incubation period.

Test Compound	S9 Mix	Endoreduplications per 2000 metaphases	% Endoreduplication	MI (%)
Eugenol (μM)				
0	+	0 ± 0	0 ± 0	6.56 ± 0.55
500	+	13.0 ± 7.0	0.65 ± 0.35	3.73 ± 1.01
1000	+	30.5 ± 7.8	1.53 ± 0.39	3.40 ± 0
1500	+	59.3 ± 10.6*	2.97 ± 0.53	2.90 ± 0.28
2000	+	101.3 ± 39.4*	5.07 ± 1.97	2.10 ± 0.44
2500	+	a	a	0.70 ± 0.42
ICRF-193 (μM)				
0	+	0 ± 0	0 ± 0	8.60 ± 1.41
0.1	+	20.5 ± 0.7*	1.03 ± 0.04	5.85 ± 0.78
0.2	+	44.0 ± 0*	2.20 ± 0	4.70 ± 0.42
0.3	+	58.0 ± 4.2*	2.90 ± 0.21	4.05 ± 0.35
0.5	+	62.0 ± 7.1**	3.10 ± 0.35	3.65 ± 0.50

[a] High levels of cytotoxicity were observed at this concentration. *$P < 0.05$ (Student's *t*-test) when compared to the control. **$P = 0.051$ (Student's *t*-test) when compared to the control.

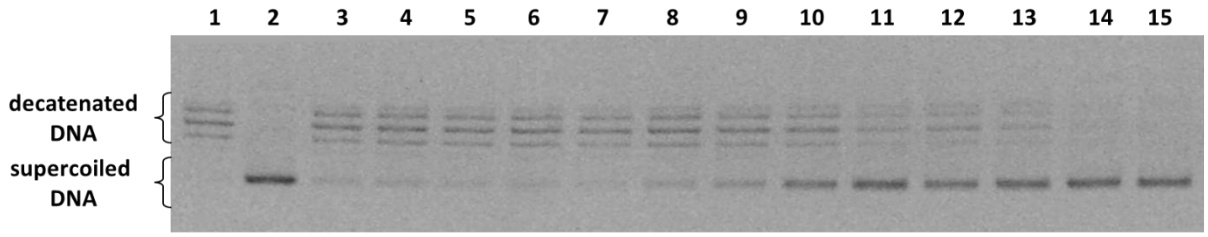

Figure 2.6 Topo II activity in the presence of eugenol or ICRF-193. Lanes 1 to 15 - pRYG (0.04 pmol), lane 1 - with Human Topo IIα (1 unit), lane 2 - without enzyme, lane 3 - with enzyme and DMSO, lanes 4 to 12 - with enzyme and eugenol (5, 10, 50, 100, 500, 1000, 2500, 5000 and 7500 μM, respectively), and lanes 13 to 15 - with enzyme and ICRF-193 (1, 5 and 10 μM, respectively).

2.4 DISCUSSION

Eugenol is a widely used flavouring substance present in various foodstuffs but also finds application in cosmetics and perfumes. Data collected on eugenol has shown that it is genotoxic in various test systems e.g. inducing CAs in eukaryotic cells (Hikiba et al., 2005; NTP, 1983) and mutations and recombination in the Drosophila wing SMART test (Munerato et al., 2005). Concern about the carcinogenicity of eugenol has risen mainly because of structural similarities with the alkenylbenzene safrole, which is classified by IARC as possibly carcinogenic to humans

(IARC, 1987), even though carcinogenicity studies by the NTP revealed equivocal results in B6C3F1 male or female mice (NTP, 1983).

Two other food alkenylbenzenes, estragole and methyleugenol, have been evaluated for their safety by the EU Scientific Committee on Food (EU-SCF, 2001a, b), classifying them as genotoxic, indicating the need for restrictions on consumption. In contrast, the industrial expert panel from the Flavour and Extract Manufacturers Association (FEMA) considered that exposure to methyleugenol and estragole does not pose a significant threat to humans (Smith et al., 2002). This conclusion is based on mechanistic insight on the biotransformation of these alkenylbenzenes and the mechanisms of genotoxicity. Thus, according to the FEMA expert panel, the profiles of metabolism, bioactivation and covalent binding of these flavours are dose dependent, with a marked decrease at lower exposure doses (1–10 mg/kg) that are 100–1000 times higher than calculated or anticipated human exposure (Smith et al., 2002).

Owing to structural similarities, the mechanisms of genotoxicity of eugenol may show similarities with those of estragole and methyleugenol. Nevertheless, these mechanisms are not fully known. In these studies we demonstrated that eugenol induces chromosome aberrations, including exchanges, in V79 cells (Figure 2.3 and Table 2.1), in particular in the presence of rat liver S9 mix, which suggests biotransformation to reactive metabolites, in agreement with published reports (Bodell et al., 1998; Hikiba et al., 2005; Thompson et al., 1989). Eugenol is known to undergo at least two major biotransformation reactions, epoxidation leading to 2´,3´-epoxyeugenol and hydroxylation with generation of 1´-hydroxyeugenol and subsequently leading to the formation of the reactive metabolite quinone methide. Since V79 cells are known to be devoid of cytochrome P450 activity (Doehmer et al., 1988; Glatt et al., 1987), the genotoxicity results in the absence of S9 mix could be due to either the direct acting activity of eugenol or possibly through formation of ROS (Sakano et al., 2004; Yoo et al., 2005). The fact that 8-hydroxy-2´-deoxyguanosine can be produced by eugenol lends credence to this latter hypothesis (Bodell et al., 1998).

Regarding CAs, we observed essentially the formation of chromatid exchanges (triradial and quadriradial figures, as we can see in the images (A) from Figure 2.3, indicating recombination phenomena that could indicate other biological activities of eugenol or its metabolites. Published

data has indicated a potential for eugenol being recombinogenic (Munerato et al., 2005). From our results, an interesting observation was the induction of ERs induced by eugenol, which increased in a dose-dependent manner (Figure 2.4), being potentiated in the presence of S9 mix. ER is a rare phenomenom observed in animal cells, frequently induced by chemicals, typically inhibitors of topo II (Cortes et al., 2004; Cortes et al., 2003a, b; Pastor et al., 2005; Pastor et al., 2002; Sumner, 1998), entailing two successive rounds of DNA replication without segregation of daughter chromatids, giving rise to diplochromosomes. The mechanisms involved in the induction of ER are not known, but probably involve DNA damage, cytoskeleton disturbance or topo II inhibition and certainly include disruption of cellular checkpoint controls (Cortes et al., 2003b).

Topo II is an essential cellular enzyme that catalyses changes in DNA topology via its cleavage–religation equilibrium, allowing decatenation of catenated DNA, which is fundamental in various cellular processes, including DNA replication and cell division (Larsen et al., 2003). Agents that are capable of stabilizing the DNA-topo II complex, also known as the cleavable complex, are called topo II poisons, whereas agents that act on other steps of the enzyme's catalytic cycle are called catalytic inhibitors (Larsen et al., 2003). Topo II targeted drugs that are poisons of this enzyme stabilize topo II–DNA covalent complexes, leading to chromosome breaks. The finding that inhibitors of topo II could induce ER (Pastor et al., 2005; Sumner, 1995, 1998) led us to evaluate the inhibitory activity of eugenol towards topo II. Our results (Figure 2.6) showed that eugenol does, indeed, inhibit the catalytic activity of topo II. Comparison of the inhibitory activity of eugenol with that of an established topo II inhibitor, ICRF-193, revealed that the latter is more potent than eugenol. ICRF-193 was chosen as a reference inhibitor since it is a known topo II inhibitor and also induced ER (Pastor et al., 2002). Our results confirm the endoreduplicating activity of ICRF-193, in V79 cells. Interestingly, the relative concentrations of eugenol and ICRF-193 required to induce ER seems to be maintained when evaluating topo II inhibition (aproximately 5000-fold difference). Lynch et al. (Lynch et al., 2003) have observed that the topo II inhibitors etoposide, doxorubicin, ciprofloxacin and genistein exhibited *in vitro* thresholds for clastogenicity using L5178Y mouse lymphoma cells. However, these cell lines are transformed and p53 deficient. The authors of the work stated that the relevance of their observations to a

genotoxic risk assessment is uncertain, as are the concentrations that define the threshold for the topo II inhibitors. Thus, caution is needed in considering a threshold approach for eugenol and related compounds since they have been shown to be genotoxic in various assays.

Former reports have indicated an increase in ER associated with a deficiency in various DNA repair genes, including the XRCC3 gene, important in recombination processes (Yoshihara et al., 2004).The Chinese hamster cell line EM9 is a repair deficient mutant that shows elevated indices of ER (Pastor et al., 2002) and a 10-fold higher baseline frequency of SCE relative to the parental cell line AA8 and also a higher sensitivity to killing by X rays. This defect is corrected by the human XRCC1 gene (Caldecott et al., 1992), once again, implicating DNA repair genes in its phenotype. Thus, further studies are required to evaluate the endoreduplicating activity of eugenol in other cell lines such as EM9 and topo II inhibition *in vivo*. In conclusion, in this report we confirm the genotoxicity of eugenol and the importance of biotransformation in its genotoxicity, in particular the induction of ER, raising the possibility of eugenol being an inhibitor of topo II.

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3. Chapter

ESTRAGOLE: A WEAK DIRECT-ACTING FOOD-BORNE GENOTOXIN AND POTENTIAL CARCINOGEN

This chapter was adapted from:

Estragole: A weak direct-acting food-borne genotoxin and potential carcinogen

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ABSTRACT

We evaluated the genotoxicity of the food-flavouring agent estragole in V79 cells using different endpoints, the CAs, the sister chromatid exchange assay (SCE) and the alkaline comet assay. Unexpectedly, we observed an increase in SCE without an exogenous biotransformation system (S9) and a decrease in its presence. Positive results were also observed in the alkaline comet assay without S9, indicating DNA strand breakage. To ascertain repair of damage, we performed the comet assay in V79 cells after two hours of recovery, and observed a reduction of the genotoxic response. Estragole did not produce strand breaks in plasmid DNA *in vitro*. We then evaluated the formation of DNA adducts in V79 cells by use of the ^{32}P-postlabelling assay and detected a dose-dependent formation of DNA adducts, which may be responsible for its genotoxicity. We then assayed estragole in the comet assay with two CHO cell lines, a parental AA8 cell line, and an XRCC1-deficient cell line, EM9. Results confirmed the genotoxicity of estragole without biotransformation in both cell lines, although the genotoxicity in EM9 cells compared with that in AA8 cells was not significantly different, suggesting that the XRCC1 protein is not involved in the repair of estragole-induced lesions. Estragole induces apoptosis, but only with high doses (2000 μM), and after long treatment periods (24 h). Overall, our results suggest that estragole, besides being metabolised to genotoxic metabolites, is a weak direct-acting genotoxin that forms DNA adducts.

3.1 INTRODUCTION

The evaluation of the carcinogenicity of the alkenylbenzene safrole led to awareness of the potential carcinogenicity of similar alkenylbenzenes, such as estragole, eugenol and methyleugenol (Miller et al., 1983; Wiseman et al., 1985; Wiseman et al., 1987). Estragole (1-allyl-4-methoxybenzene; CAS 140-67-0) is an alkenylbenzene derivative present in various

foodstuffs and is used as a flavouring substance in seasonings, condiments, and anise beverages. It is also used in teas to prevent flatulence and spasms. Human exposure to estragole occurs essentially through consumption of the spices tarragon, basil, anise and estragole-derived essential oils, reaching an average intake of 71 µg/kg bw/day (97.5th percentile reaching 141 µg/kg bw/day) (EU-SCF, 2001). An estimate of the total intake from all sources was established at 1 mg/person per day (Coe, 2006). However, in infants, exposure could be higher. According to Raffo et al. (Raffo et al., 2011), estimated exposure in infants just from teas could be up to 51 µg/kg bw/day. Estragole is approved by the U.S. Food and Drug Administration (FDA) for use in foods. The expert panel of the Flavor and Extract Manufacturers Association of the U.S. (FEMA) concluded that exposure to estragole and methyleugenol from flavours does not pose a significant cancer risk for humans (Smith et al., 2002), essentially because rodent studies have shown that the activating biotransformation pathway is less important at low doses. However, the EU Scientific Committee on Food (EU-SCF, 2001) has considered this substance to be genotoxic and carcinogenic. Based on this recommendation, the European Commission banned estragole as a food additive and established maximum levels for the use of natural flavourings and/or food ingredients containing estragole (EC, 2008). The report by the U.S. National Toxicology Program (NTP) on the 3-month toxicity study of estragole showed that oral exposure of F344/N male rats to 600mg/kg bw caused cancer of the liver (multiple cholangio-carcinoma and hepatocellular adenoma) in three of 10 animals. In that study other, non-neoplastic lesions were observed in liver, stomach and kidney (Bristol, 2011). The fact that neoplastic lesions were observed after just a three-month exposure period raises questions about the health risk associated with consumption of estragole.

Genotoxicity studies have shown negative results in most tests. Also, in various assays estragole was not clearly mutagenic in the Ames *Salmonella typhimurium* strains (EU-SCF, 2001). Convincing evidence of genotoxicity *in vitro* comes essentially from the adduct data (Zhou et al., 2007) and from the unscheduled DNA synthesis assay (Chan and Caldwell, 1992; Muller et al., 1994). To the best of our knowledge, the sister chromatid exchange (SCE) assay or the single-cell gel electrophoresis (alkaline comet) assay have not been used to evaluate the genotoxicity of estragole.

Further data on the mechanisms of genotoxicity of estragole and other structurally related flavourings, such as eugenol, methyleugenol and myristicin, should help in evaluating the health hazards associated with their consumption. In addition, the increasing consumption of so-called natural plant extracts containing these compounds reinforces the need to acquire more information on their genotoxicity, which could help in risk evaluation (Raffo et al., 2011).

In keeping with our interest in the genotoxicity of flavourings (Maralhas et al., 2006; Marques et al., 2002; Martins et al., 2011; Silva et al., 2000) our goal was to evaluate the genotoxicity of estragole and the mechanisms that may modulate its DNA-damaging activity, namely by gaining insight in the reparability of estragole-induced DNA lesions and the ability of estragole to induce apoptosis. We report that estragole induces SCE in the absence of biotransformation, suggesting that DNA lesions were formed. We confirmed this with the alkaline version of the comet assay, an indicator of DNA damage (Olive and Banath, 2006) and with the ^{32}P-postlabelling assay for DNA adducts. In addition, we were interested in evaluating the genotoxicity of estragole in a repair-deficient *XRCC1*$^{-}$ CHO cell line (EM9) (Caldecott and Thompson, 1994; Caldecott et al., 1992) to gain insight in the repair of DNA lesions in the comet assay and the induction of apoptosis in these cells.

3.2 MATERIAL AND METHODS

3.2.1 Chemicals and Reagents

Estragole (1-allyl-4-methoxybenzene, CAS No. 140-67-0, 98%), BrdU, MTT and all the other reagents, unless otherwise specified, were obtained from Sigma-Aldrich (St. Louis, MO, USA).

Propidium iodide was obtained from Chemicon International (now Millipore; Billerica, MA). DMSO, methanol, ethanol, acetic acid, potassium chloride, sodium chloride, and Giemsa dye were obtained from Merck (Darmstadt, Germany). Colchicine (CAS No. 79-06-1, ≥99.5% pure) was purchased from Fluka (Buchs, Switzerland). Rat-liver S9 (induced with Aroclor 1254) was purchased from TRINOVA Biochem GmbH (Giessen, Germany). Vectashield mounting medium H-1000 was obtained from Vector Laboratories (Burlingame, Canada).

3.2.2 Cell Culture

The wild-type V79 Chinese hamster cells (MZ) used in this study were kindly provided by Prof. H.-R. Glatt (German Institute of Human Nutrition, Nuthetal, Germany). The AA8 and EM9 cell lines were kindly provided by Prof. Felipe Cortés (University of Seville, Spain). The cells were routinely maintained in 175-cm2 culture flasks (Sarstedt, AG&CO, Nümbrecht, Germany) in Ham's F-10 medium, supplemented with 1% antibiotic solution (penicillin-streptomycin) and 10% newborn calf serum (V79 cells) or foetal bovine serum (AA8 and EM9 cells). The cells were kept at 37ºC, under an atmosphere containing 5% CO2.

3.2.3 MTT viability assay

Approximately 6×10^3 cells were cultured in 200 µl of culture medium per well, in 96-well plates and incubated at 37ºC under a 5% CO_2 atmosphere. The cells were grown for 3 and 24 h periods and then exposed to different concentrations of estragole (dissolved in DMSO, not exceeding 0.2%), ranging from 10 to 3500 or 5000 µM, for the 3 or 24h periods, respectively. The cells were washed with culture medium and MTT (dissolved in culture medium) was added to each well at a concentration of 0.5 mg/ml. The cells were grown for a further period of 3 h and then carefully washed with PBS. DMSO (200 µl) was added to each well to dissolve the formazan crystals. Absorbance was read at 595 nm in a Zenyth 3100 microplate reader. At least three independent experiments were performed and at least four individual cultures were used for each estragole concentration in each independent experiment. Hydrogen peroxide (1 mM) was used as a positive control (data not shown) and DMSO at 0.2% (v/v) was used for the negative control (control cultures). Absorbance values presented by control cultures correspond to 100% cell viability.

3.2.4 Chromosome Aberrations Assay (CAs)

Twenty-four hour cultures (~5×10^5 cells/flask) growing as monolayers in 25-cm^2 tissue culture flasks were treated with different doses of estragole (2500 – 5000 µM) dissolved in DMSO, for 3 h. When testing in the presence of a biotransformation system, 500 µl of 10% (v/v) S9 mix,

prepared according to Maron and Ames (Maron and Ames, 1983), was added to the flasks. In all the experiments, the cells were then washed and incubated with fresh medium for an additional period of 13 h. Colchicine was then added at a final concentration of 6.5 mM. Cells were grown for a further 3 h and then harvested by trypsinization. After 3 min hypotonic treatment with KCl 75 mM at 37ºC, cells were fixed with ice-cold methanol/acetic acid (3:1) and slides were prepared and stained with Giemsa at 4% (v/v) in 10 mM phosphate buffer (pH 6.8), for 10 min. Two to three independent experiments were carried out for each dose and 100 metaphases were scored. Control cells included DMSO as negative control, which did not exceed 0.5% (v/v) of the culture medium, and MMC as positive control (MMC, 0.36 µM) in the absence of S9 mix and cyclophosphamide (CP, 21.5 µM) in the presence of S9 mix (data not shown). At least two independent experiments were performed and data are indicated as percentage of aberrant cells excluding "gaps" (% CAEG) (average ± SEM).

3.2.5 Sister chromatid exchange assay (SCE)

V79 cells (~ 5 x 105), that had been cultured for 24 h in 25-cm^2 culture flasks, were exposed to different final concentrations of estragole ranging from 250 to 1000 µM, with and without an exogenous metabolic activation system, S9-mix, prepared according to Maron and Ames (Maron and Ames, 1983). Two hours later the cells were washed with fresh culture medium and BrdU was added at a final concentration of 6 µM. After 25 h, colchicine was added at a final concentration of 600 ng/ml. The cells were then incubated for a further three hours and then harvested by trypsinization. Following a 3-min hypotonic treatment with KCl (0.56%, w/v) at 37ºC, the cells were fixed with methanol/acetic acid (3:1). As positive controls we used MMC 750 nM and CP 20 µM, for tests without and with metabolic activation, respectively. S9 mix was added at the final concentration of 10% (v/v) when required.

Differential staining of BrdU-substituted sister-chromatids was performed according to the fluorescence-plus-Giemsa (FPG) method (Perry and Wolff, 1974). Briefly, the slides were stained for 12 min with the fluorescent dye Hoescht 33258 (10 µg/ml) in 2% KCl (w/v), exposed to UV (254 nm) for approximately 9 min, and then stained with 4% Giemsa (v/v) in 10mM phosphate buffer (pH 6.8) for 10 min.

SCEs per cell were scored in 30 s-metaphases for each dose-level in each experiment. At least three or four independent experiments were performed, with and without S9 mix, respectively. The evaluation of cell proliferation was carried out using the mitotic index (MI). For this index, one thousand V79 cells were scored for each independent experiment and the number of metaphases recorded. At least 100 metaphases per culture for each dose-level, in each independent experiment, were scored for the replication index (RI), calculated according to the formula RI = (M_1+2M_2+3M_3)/100 (Krishna and Theiss, 1995), where M_1, M_2 and M_3 represent the proportion of the first, second and third metaphases, respectively.

3.2.6 Plasmid DNA strand break assay

Plasmid pUC18 DNA (2686 bp) was incubated with different concentrations of estragole (0 – 750 µM) dissolved in Tris·HCl buffer (50 mM Tris·HCl, 50 mM NaCl, 1 mM EDTA) pH 6.0, 7.2 and 8.0, at 37ºC. After a 1-h incubation period, samples containing 250 ng DNA were applied to a 0.8% (w/v) agarose gel containing ethidium bromide and run at 100 V for 40 min in TBE (89 mM boric acid, 89 mM Tris·base, 1 mM EDTA pH 8.3). The gels were examined and photographed under UV light.

3.2.7 Alkaline comet assay

The comet assay was performed under alkaline conditions essentially as described (Singh et al., 1988), with modifications as described below. Cells were incubated with estragole ranging from 100 to 750 µM final concentration, for 1 h in F-10 medium supplemented with 10% serum and 1% antibiotic solution. Cells without estragole were used as negative control, and H_2O_2 treatment at 120 µM for 1 h was used as positive control. For recovery assays, cells were washed and fresh medium was added and incubated for two more hours. After treatment, cells were washed with medium, trypsinized and re-suspended in PBS. Then, 30-40 µL of cell suspension (5000-10,000 cells) were dissolved in 0.5% low melting-point agarose, and immediately spread onto a glass microscope slide pre-coated with a layer of 1% normal melting-point agarose. Agarose was allowed to set at 4ºC for 20 min. The slides were then incubated in ice-cold lysis solution (2.5 M NaCl, 10 mM Tris, 100 mM EDTA, 1% Triton, pH 10.0) at 4ºC for 1 h. After lysis, the slides were

washed gently with fresh water and placed on a horizontal electrophoresis unit. The unit was filled with fresh buffer (10 M NaOH, 200 mM EDTA, pH >13.0) so as to cover the slides for 20 min in order to allow DNA unwinding. Electrophoresis was conducted for 20 min at 0.8 V/cm (~300 mA) at 4ºC. Slides were then neutralized (0.4 M Tris, pH 7.5), dried with ethanol and stained with ethidium bromide (20 μg/ml). Cells were analysed at a 200× magnification by fluorescence microscopy (Leica DMLB, Germany), equipped with an excitation filter of 535/550nm, a short-arc HBO 103 W/2 mercury lamp, and a barrier filter of 610/675nm attached to a digital camera (Applied imaging Corp., now Genetix) connected to a personal computer. Images of randomly selected cells were captured from each slide, by use of Cytovision (v3.0) capture software (Genetix). The percentage DNA-in-tail was measured with Tritek CometScore freeware (v1.5) (www.autocomet.com). At least 50 cells were analysed per slide (two slides per independent experience giving a total of 100 cells for each concentration per experiment), and at least two independent experiments were performed.

3.2.8 Apoptosis annexin assay

AA8 or EM9 cells were seeded in 8-well chamber slides (LABTEK, Nalge Nunc Int.). After 24 h, medium was removed and replaced with fresh culture medium containing estragole at final concentrations ranging from 50 to 2000 μM. After different treatment periods, medium was removed, and cells were washed with binding buffer (0.1 M HEPES, 1.4 M NaCl, 25 mM $CaCl_2$, pH=7.4). The topoisomerase inhibitor camptothecin (CPT) was used as a positive control (16 μg/ml). Annexin V –FITC (2 ng/ml) and propidium iodide (PI, 2 μg/ml) in binding buffer was added to each well for 20 min at 37ºC, in the dark. After this period, cells were washed with binding buffer, and Hoechst 33258 prepared in binding buffer was added (1 μg/ml) for 10 min. Cells were finally washed with binding buffer and mounted with Anti-fade mounting medium (VectaSHIELD® H-1000), and analysed by fluorescence microscope (LEICA DMLB), equipped with the appropriate filters and attached to a digital camera (Applied imaging) and to a personal computer. Images were captured from each slide using Cytovision (v3.0) capture software. Quantification of apoptotic cells was undertaken with the ImageJ freeware (Rasband, W.S., U.S. National Institute of Health, Bethesda, MD, USA, http://rsb.info.nih.gov/ij/ 1997-2008) using the nucleus-counter

macro. Duplicate assays were performed and at least three different fields were analysed per dose per experiment.

3.2.9 DNA isolation for adduct detection

Twenty-four–hour cultures, growing in 75-cm^2 culture flasks, were exposed to different final concentrations of estragole (500, 750 and 1000 µM) during two different time periods (2 and 8 h), and during a 2 h period followed by a 25 h incubation without estragole (parallel cultures of the SCE assay). After incubation, the medium was removed and cells were harvested by trypsinization. Subsequently, centrifugation at 350 ×*g* and two washing steps with PBS yielded a cell pellet, which was stored at -20ºC until DNA isolation. To isolate the DNA from V79 cells, the pellet was resuspended in 500 µl lysis buffer (10 mM Tris, 1 mM EDTA and 1% (w/v) sodium dodecyl sulphate (SDS); pH 8.0) and incubated overnight at 37ºC with proteinase K (1 mg/ml) (Roche). The mixture was extracted once with an equal volume of Tris-saturated phenol (Roti-phenol; Roth, Karlsruhe, Germany), one volume of Tris-saturated phenol-chloroform-isoamyl alcohol (25:24:1, w/w) (Roti-phenol/chloroform/isoamyl alcohol; Roth) and one volume chloroform-isoamyl alcohol (24:1, v/v) (Roti-C/I; Roth). The DNA was precipitated with 0.1 vol% 5 M NaCl and 2 vol% ethanol, washed with 70% ethanol, dried and dissolved in 250 µl TE buffer (10 mM Tris-HCl, 0.1 mM EDTA, pH 8.0). Samples were treated with RNase by adding 5 µl RNase stock solution (RNase A 2 mg/ml + RNase T1 200 U/ml; Roche) for 1 h at 37ºC. An equal volume (250 µl) of proteinase K buffer (20 mM EDTA, 40 mM Tris, pH 8.0) and 50 µg/ml proteinase K was added and incubation was continued for 1 h at 37ºC. The mixture was extracted again as described above; precipitated DNA was re-suspended in TE buffer (pH 8.0). The DNA samples were quantified with the use of the Quan-iT^{TM}Picogreen® dsDNA Assay Kit (Invitrogen), according to the manufacturer's recommendations.

3.2.10 ^{32}P-Postlabelling analysis

The ^{32}P-postlabelling assay was performed on 4 µg of DNA using the nuclease extraction method of sensitivity enhancement, as described previously (Phillips and Arlt, 2007). After extracts were labelled, thin-layer chromatography on PEI-cellulose was carried out using the following solvents:

D1, 2.3 M sodium phosphate, pH 6.0; D2, 1.75 M lithium phosphate, 4.25 M urea, pH 3.5; D3, 0.32 M lithium chloride, 0.2 M Tris, 8.5 M urea, pH 8.0; D4, 1.7 M sodium phosphate, pH 6.0.

3.2.11 Statistical analyses

For the comet assay, the Shapiro-Wilk and Kolmogorov-Smirnov tests were used to verify the normality of the values of the percentage of DNA in tail, for each slide. The results indicated no normal distribution around the median or mean, so for each batch of 50 cells from each slide the 75th percentile was calculated and used for the statistical analysis. One-way ANOVA was performed with a post-test for linear trend, as suggested by Lovell and Omori (Lovell and Omori, 2008).

For the SCE assay and DNA-adduct measurements, one-way ANOVA was used to analyze the dose-response curves with a post-test for linear trend. To obtain the correlation between adducts and SCE a non-linear regression analysis was applied. All statistical analyses were performed with the SPSS statistical package (version 15) (SPSS inc., Chicago, IL), and GraphPad Prism 5 (GraphPad Software, Inc).

3.3 RESULTS

3.3.1 MTT viability assay

The results on the cytotoxicity of estragole in V79 and the two CHO cell lines, *viz.* the AA8 (parental line) and the repair-deficient line EM9, after a 3 and 24 h treatment periods, are presented in Figures 3.1 and 3.2. The differences in cytotoxicity toward the three cell lines are negligible, except for the 3 h treatment period (Figure 3.1) comparing V79 cells with CHO cells. Viability in V79 cells for all concentrations was above 80%, in contrast to a decrease from ~87% to 48% viability for CHO cells.

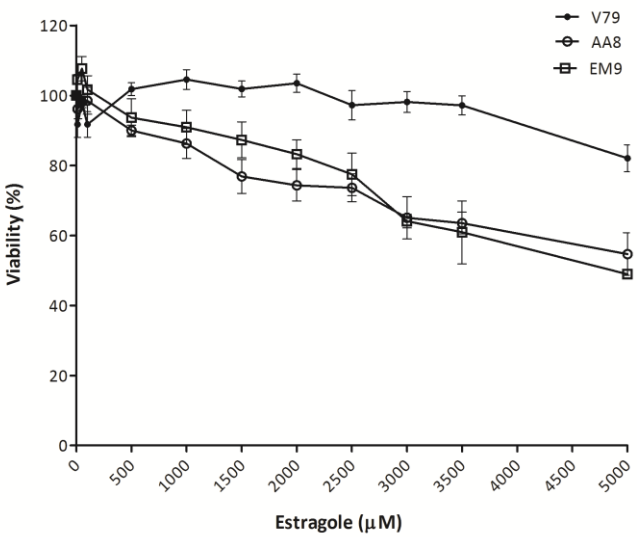


Figure 3.1 MTT viability assay in V79 and CHO cells (AA8 and EM9) exposed to estragole, after a 3h exposure period. Results are expressed as mean values ± SEM from at least four independent experiments.

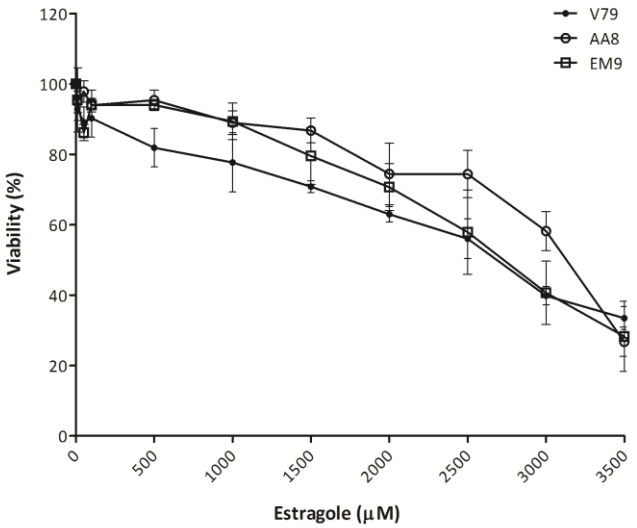


Figure 3.2 MTT viability assay in V79 and CHO cells (AA8 and EM9) exposed to estragole, after a 24 h exposure period. Results are expressed as mean values ± SEM from at least three independent experiments.

3.3.2 Cytogenetic assays (CAs and SCE)

Regarding the cytogenetic indicators studied, estragole did not induce CAs, either in the presence or absence of S9 mix, in V79 cells (Figure 3.3), in agreement with published results (Muller et al., 1994). On the other hand, estragole induced a significant dose-dependent increase of SCE in V79

cells ($P < 0.001$, one-way ANOVA; slope = 2.6), which decreased in the presence of S9 mix (slope = 0.7) (Table 3.1 and Figure 3.4). The slopes from Figure 3.4 are statistically significantly different ($P < 0.005$). Positive results observed up to 1000 µM could not be attributed to toxicity, because at these dose levels, toxicity is relatively low. Near 100% viability was observed by the MTT assay (Figure 3.1) after exposure during three hours to estragole.

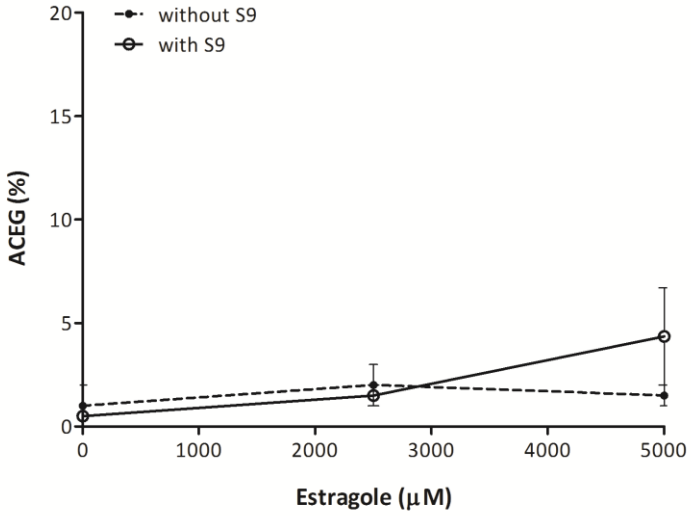


Figure 3.3 Chromosomal aberrations induced by estragole in V79 cells after a 3 h incubation period. CAs induction was measured as the percentage of chromosomal aberrant cells excluding gaps (%ACEG), in the absence and presence of a metabolic activation system S9 mix after a 3 h incubation period. Results are expressed as the average ± SEM from two independent experiments.

3.3.3 Alkaline comet assay in V79 cells

To confirm these results we performed the alkaline comet assay with estragole in V79 cells, without biotransformation. We observed a significant dose-dependent increase in the percentage of DNA in the tail of comets ($P < 0.05$, one-way ANOVA) with a significant linear trend (slope = 0.039), confirming the genotoxicity of estragole *in vitro* in the absence of an exogenous rat-liver bio-transformation system (Figure 3.5). Different batches of estragole (Sigma-Aldrich) gave similar results. In order to evaluate the persistence of DNA lesions induced by estragole, we performed the comet assay with 2 h recovery period without estragole, and the results showed a significant decrease in the dose response to estragole (Figure 3.5) (slope = 0.014). The slopes of the dose-response curves are statistically significantly different ($P < 0.05$).

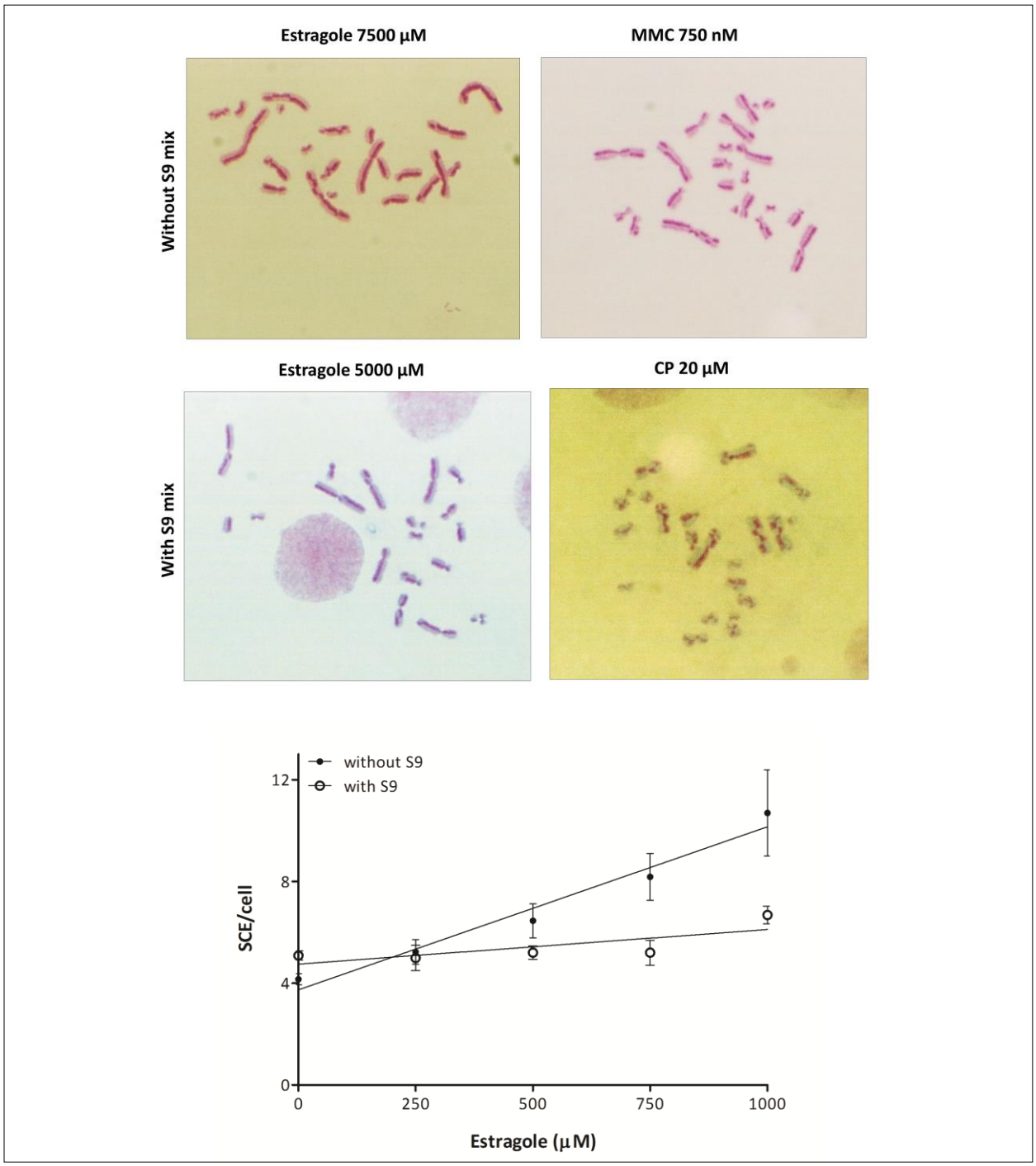


Figure 3.4 Sister chromatid exchanges induced by estragole in V79 cells. (A) Images show analysis of SCE induction in the presence and absence of S9 mix after a 3 h incubation period with estragole. The positive controls used were mitomycin C (MMC 750 nM), and cyclophosphamide (CP 20 µM), without and with S9 mix, respectively. (B) Graph values are shown as sister chromatid exchanges per cell (SCE/cell) and are expressed as the average ± SEM from four and three independent experiments, without and with S9 mix, respectively. Statistical significance was analysed using one-way ANOVA ($P < 0.001$; slope = 2.6) without S9 mix which decreased in the presence of S9 mix (slope = 0.7). The slopes are statistically significantly different ($P < 0.005$).

Table 3.1 Sister chromatid exchanges induced by estragole, with (+) and without* (–) S9 mix, in V79 cells.

Test compound	SCE /chromosome[a] (average ± SD[b])		SCE/metaphase[a] (average ± SD)		MI[c] (%) (average ± SD)		RI[d] (Average ± SD)	
	S9 mix		S9 mix		S9 mix		S9 mix	
	+	-	+	-		-	+	-
Estragole (μM)								
0.00	0.23 ± 0.01	0.19 ± 0.02	5.09 ± 0.31	4.16 ± 0.45	5.70 ± 1.04	4.53 ± 2.15	2.00 ± 0.01	1.96 ± 0.07
250	0.23 ± 0.04	0.24 ± 0.04	4.99 ± 0.86	5.23 ± 0.96	6.13 ± 1.38	6.20 ± 3.93	1.99 ± 0.03	1.97 ± 0.05
500	0.24 ± 0.02	0.30 ± 0.06	5.20 ± 0.45	6.45 ± 1.35	5.33 ± 0.58	7.00 ± 5.07	2.00 ± 0.00	1.97 ± 0.01
750	0.23 ± 0.04	0.38 ± 0.09	5.20 ± 0.85	8.18 ± 1.84	4.87 ± 0.74	5.30 ± 2.89	1.99 ± 0.01	1.93 ± 0.07
1000	0.31 ± 0.03	0.50 ± 0.16	6.68 ± 0.60	10.69 ± 3.39	5.44 ± 0.46	6.38 ± 3.61	1.98 ± 0.10	1.98 ± 0.04
MMC (μM)								
0.75	-	1.71 ± 0.68	-	36.91 ± 14.47	-	6.94 ± 2.45	-	1.97 ± 0.06
CP (μM)								
20.0	1.95 ± 0.13	-	43.03 ± 2.48	-	5.13 ± 1.12	-	1.93 ± 0.13	-

[a] The results are expressed as the average from at least three or four independent experiments, with and without S9mix, respectively (30 metaphases analyzed per dose per experiment) for all the points, [b] Standard deviation, [c] Mitotic index, [d] Replication Index. * $P < 0.001$ without S9 mix using one way ANOVA.

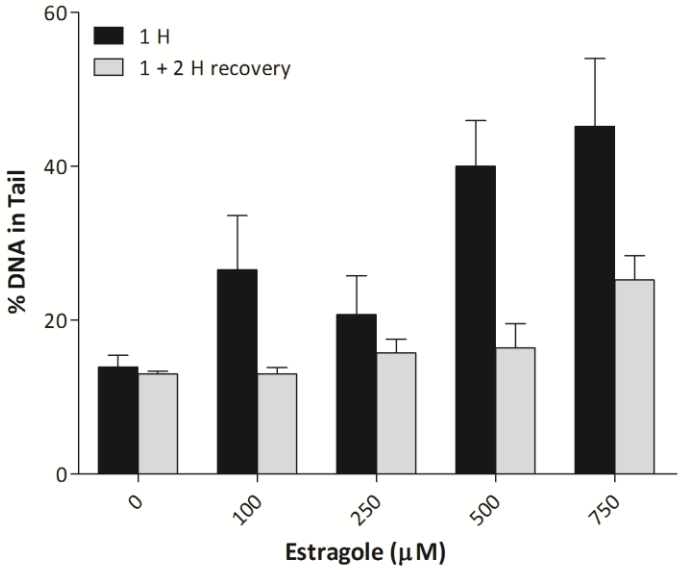


Figure 3.5 Detection of DNA damage induced by estragole in V79 cells using the alkaline comet assay. Data presented as 75th percentile (mean ± SEM) of % DNA in tail. Results are given for 1 h treatments with concentrations between 100 to 750 μM of estragole, and with a 2 h recovery period. We observed a significant dose-dependent increase in the % DNA in tail ($P < 0.05$, one-way ANOVA) with a significant linear trend (slope = 0.039). After allowing two hours for recovery a significant decrease is observed in the dose response to estragole (slope = 0.014). The slopes of the dose-response curves are statistically significantly different ($P < 0.05$).

3.3.4 Plasmid strand breakage assay

The results in the comet assay could be due to direct induction of DNA strand-breaks by estragole. To evaluate this possibility, we performed an *in vitro* assay with plasmid DNA to follow the transition of supercoiled DNA to nicked open circular or linear forms, which would be indicative of formation of breaks. This assay was performed with three pH values (6.0, 7.2 and 8.0), since with some natural compounds the formation of ROS occurs with different pH values (Gaspar et al., 1994)**.** However, the results were negative (Figure 3.6) indicating that the strand-breaks viewed in the comet assay are cell-dependent.

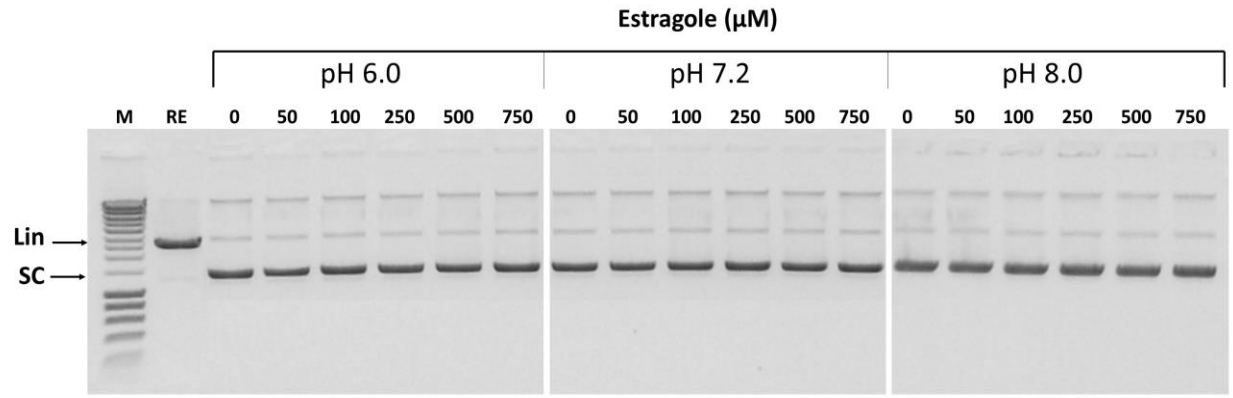


Figure 3.6 Agarose-gel electrophoresis of plasmid DNA after incubation with different concentrations of estragole (µM) and at various pH values (6.0, 7.2 and 8.0). DNA (250 ng) was loaded into each well. No DNA strand-breakage was observed under any of the assay conditions. (M, molecular weight marker; Lin, linear DNA; SC, supercoiled DNA; RE, linearized plasmid DNA after hydrolysis with *Pst*I restriction enzyme). Data are from one of two experiments with similar results, after a 1 h exposure period to estragole.

3.3.5 Adduct formation by estragole in V79 cells

Published results indicate that estragole can form DNA adducts after biotransformation *via* the 1'-sulfooxyestragole route (Phillips et al., 1981). Since we observed a dose-dependent increase in DNA damage in V79 cells, we evaluated the possibility of adduct formation by estragole without exogenous bio-transformation. Results indicate a consistent increase in adduct levels (per 10^8 nucleotides), Figure 3.7 (B), after a 2 h incubation with estragole. The thin-layer chromatograms revealed a single adduct spot, images I and II from Figure 3.7 (A), this adduct spot was not visibly detectable in those of DNA from control cells. Incubation of V79 cells for

eight hours, or two hours plus 25 h incubation without estragole, image II from Figure 3.7 (A), did not alter the dose-response curve as we can see in Figure 3.7 (B). One-way ANOVA analysis of the results for eight hours and '2 + 25 h' showed statistical significance ($P < 0.05$), and the post-test for linear trend was also statistically significant for these treatment periods ($P < 0.05$). We also determined if there was a correlation between SCE and DNA adducts formation and obtained a strong correlation of 0.962, as we can see in figure 3.9.

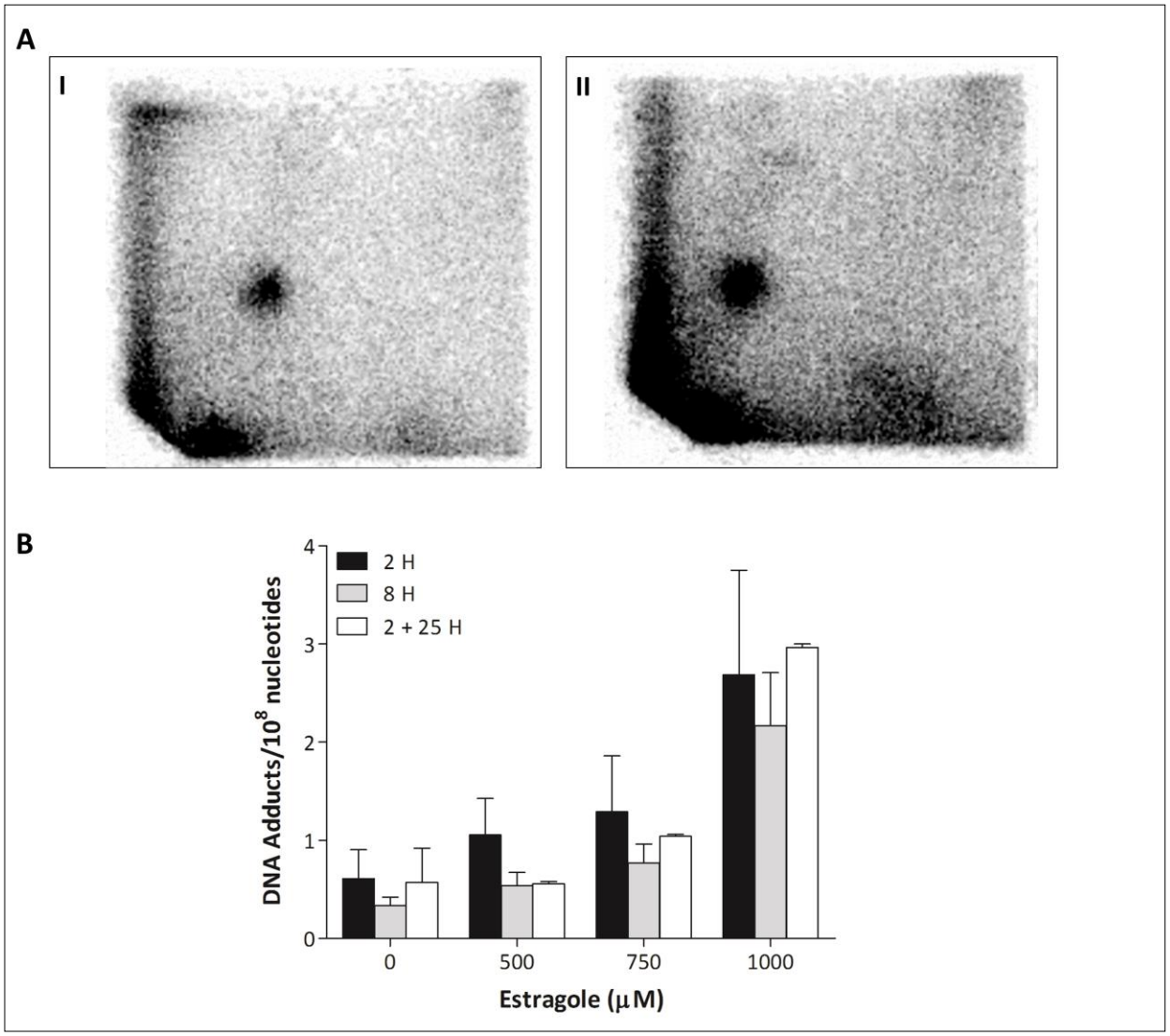


Figure 3.7 ^{32}P-postlabelling quantitative analysis of DNA adducts in V79 cells treated with estragole. V79 cells were performed after 2 and 8 h treatment periods and with a 2 h incubation with estragole plus 25 h post-incubation in culture without estragole. (A) Autoradiograph of DNA adducts. I -Estragole 1 mM, after a 2 h treatment period, II - Estragole at 1 mM, after a 2 h treatment period plus 25 h post-incubation in culture without estragole. (B) Results are expressed as the mean ± SEM from at least two independent experiments. Significant results were obtained using one-way ANOVA for 8 h treatment ($P = 0.0096$; linear trend, $P = 0.0027$) and 2+25 h treatment ($P = 0.0018$; linear trend, $P = 0.0006$).

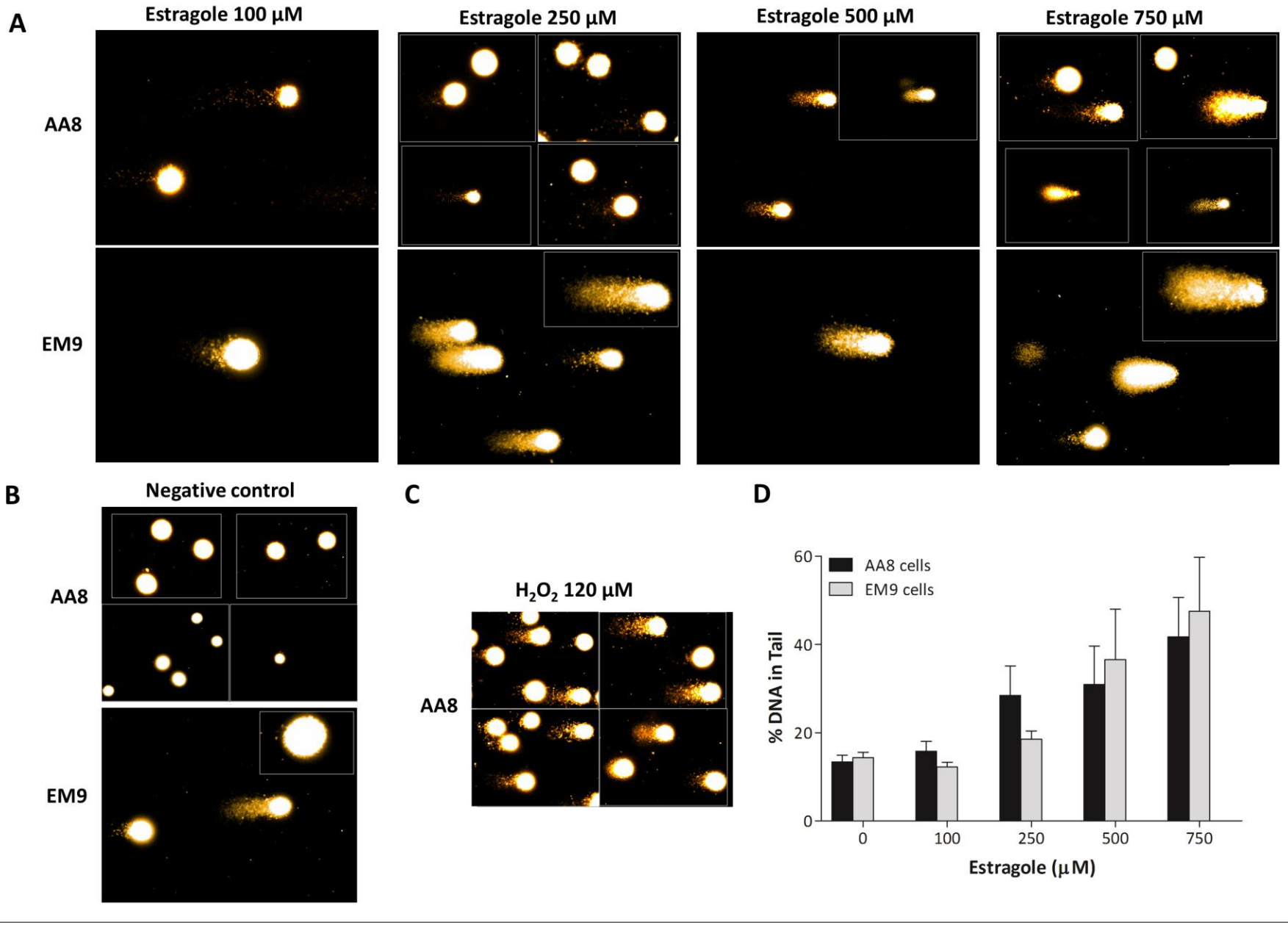

A
Estragole 100 µM
Estragole 250 µM
Estragole 500 µM
Estragole 750 µM
AA8
EM9
B
Negative control
AA8
EM9
C
H_2O_2 120 µM
AA8
D
AA8 cells
EM9 cells
% DNA in Tail
60
40
20
0
0
100
250
500
750
Estragole (µM)

Figure 3.8 Detection of DNA damage induced by estragole in AA8 and EM9 cells with the alkaline comet assay. (A) Images show analysis of comets acquired using fluorescence microscopy of AA8 and EM9 cells after exposure to 100, 250, 500 and 750 µM of estragole. (B) Negative controls, cells exposed to DMSO. (C) Positive control (H_2O_2 120 µM) in AA8 cells. (D) Graph values presented as the 75th percentile (mean ± SEM) of % DNA in tail. Results are given for 1 h treatments with concentrations between 100 and 750 µM estragole. A significant dose-dependent increase is observed in the % DNA in tail of comets ($P < 0.05$, one-way ANOVA) with a significant linear trend for both cell lines ($P < 0.005$; slope = 0.037 for AA8 *vs* 0.049 for EM9).

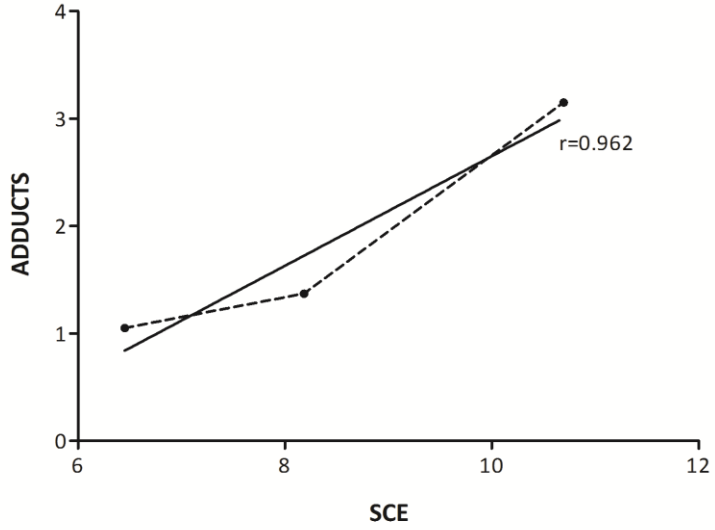
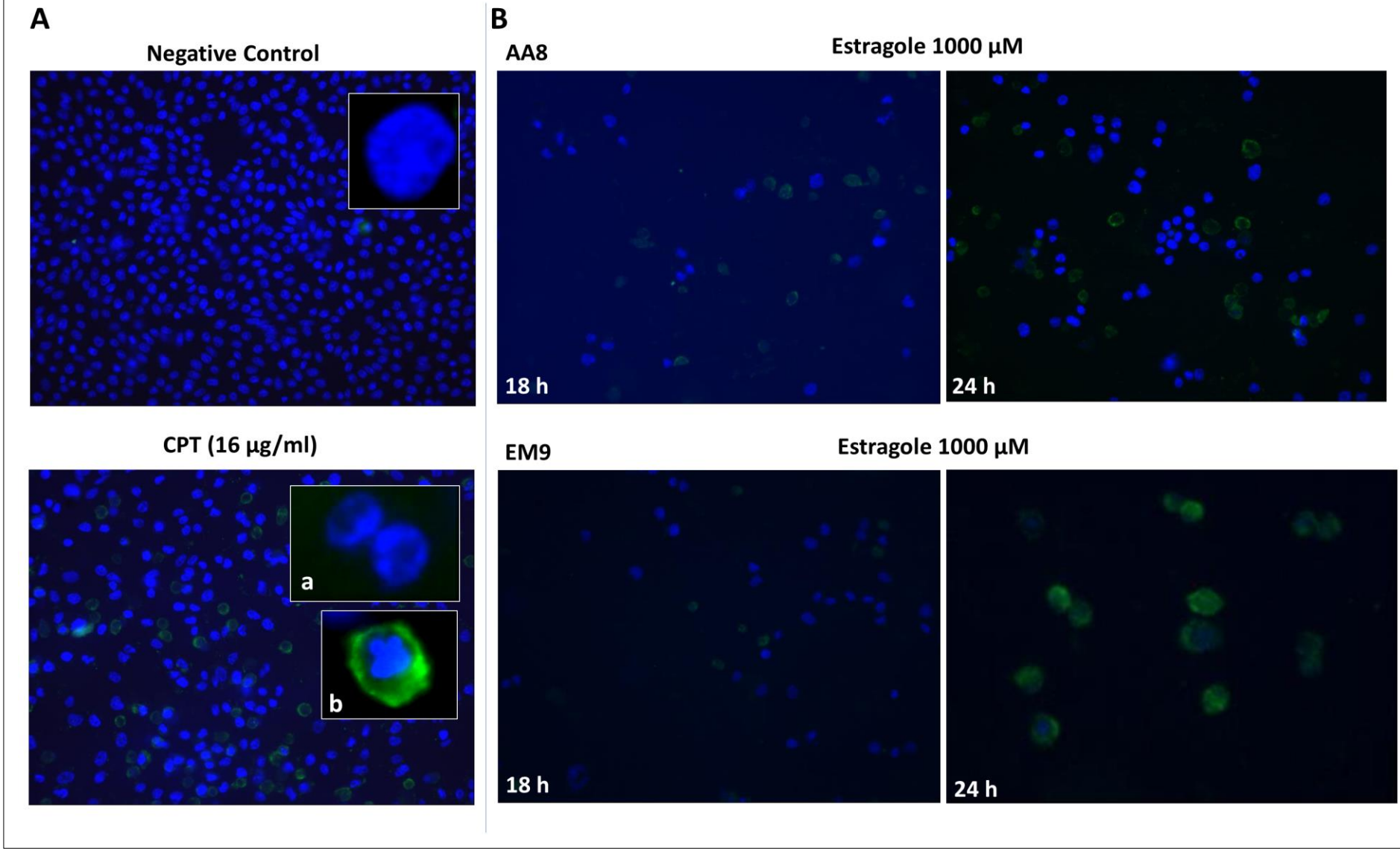


Figure 3.9 Correlation between DNA adducts and SCE in V79 cells.

3.3.6 Alkaline comet assay in AA8 and EM9 cells

To gain some insight on the repair of the DNA lesions induced by estragole, we performed the alkaline comet assay in CHO AA8 cells, and in an XRCC1$^-$ derived EM9 cell line (Caldecott et al., 1992). The XRCC1 protein has no recognizable catalytic activity, but is considered as a scaffold for protein complexes involved in for base-excision repair (BER), nucleotide-excision repair (NER) and SSB. In both cell lines estragole induced significant dose-dependent increases in the percentage tail- DNA ($P < 0.05$, one-way ANOVA) with a significant linear trend for both cell lines ($P < 0.005$; slope = 0.037 for AA8 *vs* 0.049 for EM9) (Figure 3.8). There was no significant difference when comparing AA8 with EM9 cells, suggesting that in these conditions the XRCC1 protein is not actively involved in the repair of DNA lesions.


A
Negative Control
CPT (16 µg/ml)
a
b
B
AA8
Estragole 1000 µM
18 h
24 h
EM9
Estragole 1000 µM
18 h
24 h

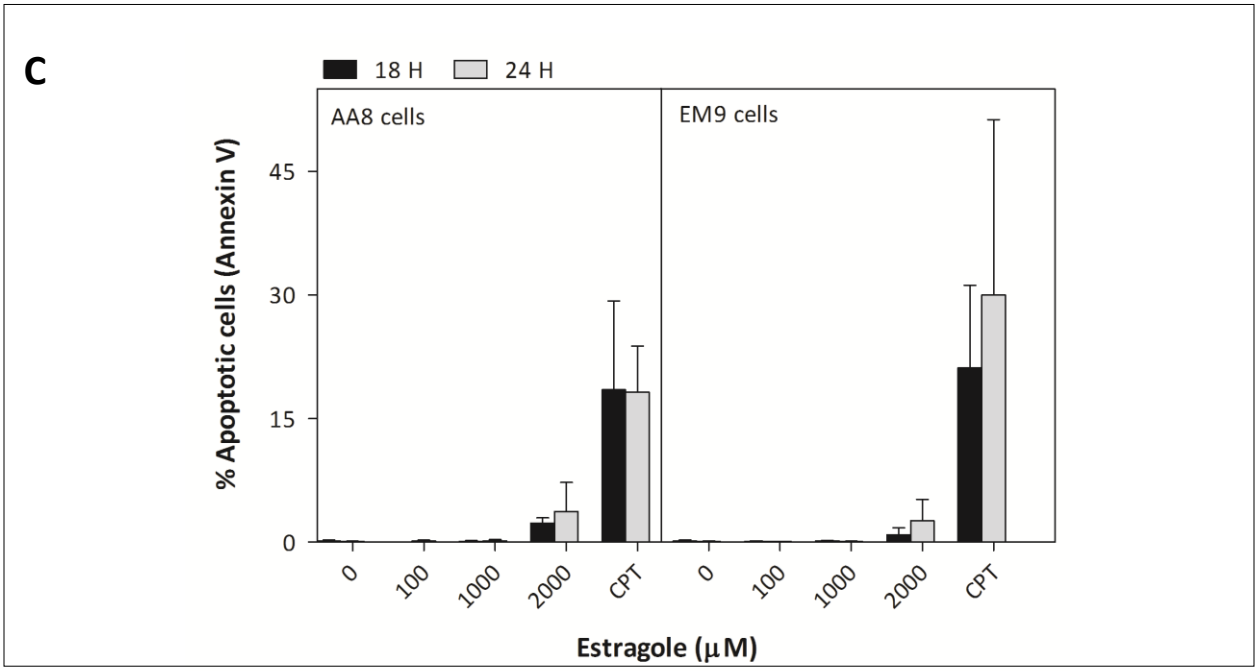


Figure 3.10 Detection of apoptosis induced by estragole in AA8 cells and EM9 cells by the annexin V assay. Cells were stained with Hoechst 33342/Annexin-V-FITC, and exposed to estragole to a 18 h and 24h incubation periods. (A) Merge images captured by fluorescence microscopy of CHO cells exposed to the negative control only with DMSO and the positive control camptothecin (CPT); a - apoptotic cells labeled with Hoechst stain, b – merge image (Hoechst 33342/Annexin-V-FITC) of an apoptotic cell. (B) Merge images captured by fluorescence microscopy of CHO cells exposed to 1000 µM of estragole. (C) Graph results are expressed as the mean ± SEM of duplicate experiments. At least three different fields were analyzed per dose and per experiment. Negative results were always obtained up to 1 mM for both cell lines and with the different incubation periods.

3.3.7 Apoptosis assay in AA8 and EM9 cells

Since the genotoxicity of estragole could influence cell death, we finally evaluated the induction of apoptosis in the AA8 and EM9 cell lines, using the annexin V assay. Our results indicate that estragole only induces apoptosis after long incubation periods (exceeding 18 h) and only with the highest dose tested (2000 µM), Figure 3.9 (C). In images A from Figure 3.9, we can see differences between the negative control and the positive control, with an emphasis on the images of the positive control (a) and (b), where we can see an apoptotic cell with Hoechst (a) and merged with Hoechst/Annexin V-FITC (b). After an 8 or 12 h incubation period apoptosis was not observed (data not shown). We also performed a pan-caspase assay to evaluate activation of caspases, but the results were essentially negative after incubation periods of up to 24 h (data not shown). No

differences were observed comparing the AA8 with EM9 cells regarding the induction of apoptosis with these markers.

3.4 DISCUSSION

Estragole and the other alkenylbenzenes safrole and methyleugenol, are known rodent carcinogens (Bristol, 2011; Miller et al., 1983; Wiseman et al., 1987). Concern about their hazard for human health has prompted a number of studies regarding their mechanisms of carcinogenicity.

The mechanism of carcinogenic action of estragole is seen to proceed *via* metabolism by cytochromes P-450 to produce 1'-hydroxyestragole. Jeurissen et al. (Jeurissen et al., 2007) identified cytochrome P450 (CYP) 1A2 and CYP2A6 as the most important enzymes in the 1'-hydroxylation of estragole in *in vitro* studies, but other CYPs can also bio-activate estragole, including CYP 1A2, 2A6, 2C9, 2C19, 2D6, and 2E1. A further bio-transformation step *via* sulfate conjugation of the 1'-hydroxyestragole metabolite by sulfotransferase (SULT) enzymes has been implicated as the most important, giving rise to an electrophilic metabolite of estragole, 1'-sulfooxyestragole, which forms adducts with DNA and proteins (Paini et al., 2010; Phillips et al., 1981; Phillips et al., 1984; Punt et al., 2007; Randerath et al., 1984; Rietjens et al., 2005; Wiseman et al., 1985). Several DNA adducts have been identified (Phillips et al., 1981). The major adduct found was N^2-(*trans*-isoestragole-3'-yl)-2'-deoxyguanosine (E-3'-N^2-dGuo), which could play an important role in the genotoxic and carcinogenic effects of estragole (Phillips et al., 1981). The importance of SULT in the bio-activation not only of estragole, but also of other alkenylbenzenes such as safrole, has been evaluated *in vitro* and also *in vivo*. For example, pentachlorophenol, an inhibitor of SULT, decreased the formation of SCE and CAs and led to a decrease of DNA adducts formed by safrole *in vivo* in rats, after a dose of 500 mg/kg bw (Daimon et al., 1997). A recent study with *O*-acetyltransferase-deficient *S. typhimurium* strains genetically engineered to express human *SULT1A1*, *1A2*, and *1A3* genes were used to evaluate the genotoxicity of estragole in the presence of S9. Estragole and 1'-hydroxylsafrole showed positive responses only in the strain expressing SULT1A3, but not in those expressing SULT1A1 or 1A2, indicating that SULT1A3 is

probably the major sulfotransferase involved in the bio-activation of alkenylbenzenes (Oda et al., 2012). Interestingly, in a study of 28 human liver samples by quantitative immunoblotting, SULT1A3 was not found to be expressed in this tissue (Riches et al., 2009), whereas it was expressed in samples of the small intestine, raising the issue of tissue-specific bio-transformation of estragole in humans.

Thus, reactivity of metabolites towards DNA is considered a major step in the carcinogenicity of estragole. The importance of CYPs and SULT in formation of the ultimate reactive metabolite could explain the negative results obtained in most *in vitro* genotoxicity assays (EU-SCF, 2001; Muller et al., 1994), since in most cell lines, either CYPs or SULT are lacking. In our study, Figure 3.3, estragole did not induce CAs in V79 Chinese hamster cells in the presence or absence of exogenous biotransformation systems as already demonstrated by Müller et al. (Muller et al., 1994). To the best of our knowledge, this is the first study reporting data from SCE or the alkaline comet assay and the positive results obtained by us in V79 cells show that estragole can be genotoxic in the absence of a metabolic activation system (S9 mix), albeit at high doses.

The positive results obtained with SCE could suggest formation of DNA adducts. Various authors have shown that adduct-forming compounds are good inducers of SCE and have shown a correlation between the levels of DNA adducts and the induction of SCE (Daimon et al., 1997; Martins et al., 2007), presumably through recombination DNA-repair mechanisms (Wilson and Thompson, 2007). The negative results for SCE in the presence of S9 could be explained by the predominance of detoxification reactions occurring with S9, although it can be difficult to clearly determine the contribution of an individual enzyme in the S9 mix to the metabolic activation of a compound (Luo and Guenthner, 1995, 1996; Oda et al., 2012).

Thus, to evaluate the formation of DNA adducts by estragole in the absence of an exogenous metabolic activation system, we performed the ^{32}P-postlabelling assay in V79 cells without S9 and we found low levels of adducts, in particular at the highest dose tested, 1000 μM (Figure 3.7). Zhou et al. (Zhou et al., 2007) used the ^{32}P-postlabelling assay on DNA from HepG2 cells and found DNA-estragole adducts. Importantly, and in line with results obtained in rodents by Randerath et al. and Phillips et al. (Phillips et al., 1984; Randerath et al., 1984), data obtained by Zhou et al. (Zhou et al., 2007) showed that estragole was more potent in adduct formation than

safrole, with an almost 7-fold higher level of formation of the major estragole adduct, relative to safrole, at 450 μM. HepG2 cells are considered metabolically competent (Knasmuller et al., 2004; Knasmuller et al., 1998)**,** although expressing very low levels of CYP transcripts (e.g. CYPs 1A1, 1A2, 2B6, 2C9, 2D6, 2E1, 3A4) and very low enzymatic activity (Boehme et al., 2010; Westerink and Schoonen, 2007a; Wilkening et al., 2003).On the other hand, HepG2 cells express a near complete set of Phase-II enzymes, including SULT1A1, 1A2, 1E1 and 2A1 (Westerink and Schoonen, 2007b). Therefore formation of reactive metabolites is possible and the adducts found in HepG2 cells were probably adducts formed from 1′-sulfooxyestragole. Conversely, V79 cells have negligible cytochrome P-450 or sulfotransferase activity (Glatt et al., 1990), therefore hydroxylation by these enzymes, if present, probably occurs at very low levels.

Nevertheless, the fact that we found low levels of DNA adducts, with a significant dose response up to 1000 μM, suggest the possibility of a direct-acting mechanism of adduction. It is not known if the adducts we found are the same as those found in other studies *in vitro* or *in vivo*, and for this further studies are needed. *In vivo* bio-transformation of estragole varies with dose, such that increasing doses in rats, for example, leads to a shift from *O*-demethylation, a potentially detoxification route, to 1′-hydroxylation, which is a toxification route (Punt et al., 2008). In addition, species differences in bio-transformation of estragole also exists, such that liver extracts of male rats are more efficient in sulfonation of the 1′-hydroxyestragole metabolite than human livers extracts, whereas in male mice the efficiency is similar (Punt et al., 2007).

Studies by Zhou et al. (Zhou et al., 2007) indicated that estragole produced a much higher level of DNA adducts when compared to safrole. Yet estragole displays the same efficiency as safrole as a rodent carcinogen [TD_{50}, the daily dose rate in mg/kg body weight/day sufficient to induce tumours in half of test animals, is 51.3 for safrole and 51.8 for estragole in mice] (Berkeley Carcinogenic Potency Database – CPDB) (Gold et al., 1991; Zhou et al., 2007). These results could be due to differences in the persistence of DNA adducts. Published data (Phillips et al., 1981) showed that DNA adduct derivatives of 1′-hydroxyestragole were removed from mouse liver after a few days, suggesting repair of adducts *in vivo*. Therefore we were interested in evaluating the persistence of the DNA lesions detected by the comet assay. In V79 cells, a clear reduction of the comet tail was observed after a 2 h recovery period, suggesting repair of DNA lesions. The

fact that the XRCC1$^-$ deficient cell line EM9 responds equally well to estragole in the comet assay compared with parental AA8 cells indicates that the XRCC1 protein is probably not relevant in the repair of lesions induced by estragole. Experiments were also conducted to evaluate the persistence of DNA adducts produced by estragole in V79 cells, after a 25 h recovery period. Our results indicate that adducts are still present after this recovery period (Figure 3.7 (A), image II), suggesting that at these levels (1000 μM) repair is not efficient.

Our results also indicate that estragole did not induce apoptosis (Figure 3.9) in all the assays performed for all concentrations tested, except for the highest concentration of 2000 μM. For this dose and a 24 h period estragole induced apoptosis to a limited extent, compared with the positive control. The MTT assays also show no significant cytotoxicity (above 50% cellular viability). We conclude that estragole does not induce apoptosis at physiologically relevant doses.

Another possible mechanism of genotoxicity *in vitro* that could explain our results is through an oxidative pathway with production of ROS, which, in the presence of antioxidant enzymes present in S9 (such as SOD, catalase, *etc.*) would be scavenged (do Ceu Silva et al., 2003; Rueff et al., 1992; Wiseman et al., 1987). However, ROS are not considered to be efficient inducers of SCE. Conversely, ROS are efficient inducers of CAs, which would imply a positive response in the CAs assay, which was not observed in V79 cells (Figure 3.3). Therefore, the involvement of ROS in the genotoxicity of estragole is doubtful. Confirming this analysis are the results of the *in vitro* plasmid DNA-breakage assay, which were negative.

In summary, according to the results obtained, it seems that genotoxicity of estragole *in vitro* for high doses may ensue in part from direct adduction of DNA which can lead to alkali-labile sites in DNA, resulting in tails in the comet assay, and SCE, due to DNA strand-breaks. Nevertheless, the doses necessary to induce a genotoxic response are far from physiologically relevant human doses, and therefore the relevance of these adducts for tumour induction in humans *in vivo* needs to be further clarified.

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4. Chapter

GENOTOXIC AND APOPTOTIC ACTIVITIES OF THE FOOD FLAVOURINGS MYRISTICIN AND EUGENOL IN AA8 AND XRCC1 DEFICIENT EM9 CELLS

This chapter was adapted from:

Genotoxic and apoptotic activities of the food flavourings myristicin and eugenol in AA8 and XRCC1 deficient EM9 cells

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ABSTRACT

Some food flavourings, such as safrole and methyleugenol, are known for their genotoxic and hepatocarcinogenic properties whereas for others, such as myristicin, there is less data. Myristicin and eugenol are both alkenylbenzenes, and we compared their direct genotoxicity in repair proficient (AA8) and repair deficient XRCC1$^-$ (EM9) Chinese hamster ovary cells. Cell viability was assessed by the MTT assay. The comet assay was used to evaluate DNA breaks, and the γ-H2AX assay to evaluate induction of double strand breaks. We assessed apoptosis by measuring caspases activation, and using the TUNEL assay. Reduction of cell viability was similar in AA8 and EM9 cells for eugenol, but myristicin was less cytotoxic in EM9 cells. After 1 h eugenol produced DNA strand breaks in the comet assay and induced double strand breaks in the γ-H2AX assay in AA8 cells, while myristicin was not genotoxic in both the comet and the γ-H2AX assays. Both flavourings were negative in EM9 cells, although myristicin had a non-significant higher response for EM9 cells. After 24 h eugenol and myristicin induced DNA fragmentation detected by TUNEL in both cell lines, but only myristicin activated caspases. Myristicin was more apoptotic than eugenol, in both cell lines. Our results regarding caspases activation indicate a higher response for myristicin in EM9 cells. In conclusion eugenol was genotoxic as measured by the comet assay and the γ-H2AX assay, while myristicin was not genotoxic but more apoptotic as measured by the caspases activation assay and the TUNEL assay. The XRCC1 protein seems to have some role in the activity of myristicin but not for eugenol.

4.1 INTRODUCTION

The consumption of natural compounds as phytochemicals or in functional foods to enhance health is increasing in interest (Aggarwal et al., 2008; Anand et al., 2008; Bakkali et al., 2008;

Clifford, 2000; Rietjens et al., 2008). Dietary components, including flavourings and additives should thus be properly studied to improve risk evaluation. Some flavourings, namely alkenylbenzenes, such as safrole, methyleugenol and estragole, are present in spices, essential oils and teas and are known for their genotoxic and carcinogenic properties. As such they are classified by the EU Scientific Committee on Food (EU-SCF) with restrictions on their use (EU-SCF, 2001a, b, 2002). Safrole is classified by the International Agency for Research on Cancer (IARC) as a class 2B carcinogen (IARC, 1987). Other alkenylbenzenes, on the other hand, such as myristicin and eugenol, have been less studied and thus less data is available on their genotoxic or carcinogenic properties. Myristicin (1-allyl-5-methoxy-3,4-methylenedioxybenzene; CAS Nº 607-91-0) and eugenol (1-allyl-3-methoxy-4-hydroxybenzene; CAS Nº 97-53-0), are found in basil, anise, cinnamon, clove, fennel, nutmeg, parsley, star anise (Bakkali et al., 2008; NTP, 1983) and in some essential oils of clove, marjoram, bay leaf and cinnamon leaf (NTP, 1983; Slamenova et al., 2009). They are also used as fragrances in the cosmetic and pesticide industry and as flavouring agents, while eugenol is also widely used in dentistry as a cement material with zinc oxide or as a sedative agent (Hikiba et al., 2005; Slamenova et al., 2009). Myristicin is also used in traditional medicine to treat rheumatism, cholera, psychosis, stomach cramps, nausea, diarrhea, flatulence, and anxiety (Barceloux, 2008).

In our previous chapter we showed that eugenol induces CAs and endoreduplication in V79 Chinese hamster fibroblasts (chapter II). The induction of CAs could be due to direct formation of single (SSB) or DSB in DNA, for example by formation of ROS, or by repair processes involving base excision repair (BER), via abasic site incision activity following removal of damaged bases, or nucleotide excision repair (NER). Given the similar structures of eugenol and myristicin, and following our previous work on the genotoxicity of food compounds (Maralhas et al., 2006; Martins et al., 2007; Oliveira et al., 2009), our objective in this study was to compare their direct genotoxicity, repair and apoptotic activities in mammalian cells namely in DNA repair-proficient AA8 Chinese hamster cells, as well as repair-deficient ($XRCC1^-$) EM9 cells. The XRCC1 protein is greatly reduced or absent in EM9 cells and plays a major role in BER and facilitating the repair of SSBs in mammalian cells, via an ability to interact with multiple enzymatic components of repair reactions (Caldecott, 2003). But XRCC1 can also be involved in double strand breaks repair (DSB)

(Xu et al., 2014). First we assessed the cytotoxicity of both compounds in both cells lines by the MTT assay. Because SSBs are induced by reactive molecules such as ROS or as a consequence of DNA repair processes, we performed the single cell gel electrophoresis assay (alkaline comet assay) in both cells lines. To detect DSBs induced by these compounds, we also performed the phosphorylated histone H2AX (γ-H2AX) detection assay. Genotoxicity assays were performed after 1 h incubation periods. We also evaluated apoptosis induced by both compounds, after a 24 h incubation period, as a possible outcome of their genotoxic activity, using the TdT-mediated dUTP-digoxigenin nick end labeling (TUNEL) assay and with detection of caspases activation.

4.2 MATERIAL AND METHODS

4.2.1 *Chemicals and Reagents*

Eugenol (CAS Nº 97-53-0), myristicin (CAS Nº 607-91-0), MTT and all the other reagents, unless otherwise specified, were obtained from Sigma-Aldrich (St. Louis, MO, USA). DMSO, ethanol, sodium chloride, and sodium hydroxide were obtained from Merck KGaA (Darmstadt, Germany). Formaldehyde solution (w/v) was obtained from Thermo scientific (Rockford, USA). Vectashield mounting medium H-1000 was obtained from Vector Laboratories (Burlingame, Canada).

4.2.2 *Cell Culture*

Chinese hamster ovary AA8 and EM9 cell lines were kindly provided by Prof. Felipe Cortés (University of Seville, Spain). The cells were routinely maintained in 175 cm^2 culture flasks (Sarstedt, AG&CO, Nümbrecht, Germany) using Ham's F-10 medium, supplemented with 10% fetal bovine serum and 1% antibiotic solution (penicillin-streptomycin) (Sigma-Aldrich, St. Louis, MO, USA). Cells were kept at 37 ºC, under an atmosphere containing 5% CO_2.

4.2.3 *MTT viability assay*

Approximately 7.5×10^3 cells were cultured in complete medium growing in 96-well plates and incubated at 37 ºC under a 5% CO_2 atmosphere. The cells were allowed to grow for twenty-four

hours and then exposed to different concentrations of eugenol or myristicin (dissolved in DMSO, not exceeding 0.2%), ranging from 50 to 2000 µM, for 3 and 24 h periods. DMSO at 0.2% (v/v) was added to the wells without chemical (control cultures). After each treatment cells were washed with culture medium and MTT was added to each well at a concentration of 0.5 mg/ml. Cells were grown for a further period of 3 h and then carefully washed with PBS. At the end of the incubation period, the media was discarded and DMSO (200 µl) was added to each well to dissolve the formazan crystals. Absorbance was read at 595 nm in a Zenyth 3100 microplate reader. Absorbance values presented by control cultures, correspond to 100% cell viability. At least two and three independent experiments were performed, for 3 and 24 h periods respectively. Eight replicates were used in each independent experiment.

4.2.4 *Alkaline comet assay*

The comet assay was performed under alkaline conditions essentially as described by Singh et al. (Singh et al., 1988), with modifications as will be described. Cells were incubated with concentrations of eugenol and myristicin, ranging from 50 to 500 µM, for 1 h in F-10 medium supplemented with 10% fetal bovine serum and 1% antibiotic solution, at 37ºC and 5% CO_2. Cells with DMSO were used as negative control, and 200 µM H_2O_2 treatment was used as a positive control. After the incubation period, cells were washed with medium, trypsinized and resuspended in PBS. Thirty to forty microliters of cell suspension (5000 - 10,000 cells) was then dissolved in 0.5% low-melting point agarose, and immediately spread onto a glass microscope slide pre-coated with a layer of 1% normal melting point agarose. Agarose was allowed to set at 4ºC for 20 min. The slides were then incubated in ice cold lysis solution (2.5 M NaCl, 10 mM Tris, 100 mM EDTA, 1% Triton, pH 10.0) at 4ºC for 1 h. After lysis, the slides were washed gently with fresh water and placed on a horizontal electrophoresis unit. The unit was filled with fresh buffer (10 M NaOH, 200 mM EDTA, pH >13.0), covering the slides for 20 min in order to allow DNA unwinding. Electrophoresis was conducted for 20 min at 0.8 V/cm (~ 300 mA) at 4ºC. Slides were then neutralized (0.4 M Tris, pH 7.5), dried with ethanol and stained with ethidium bromide (20 µg/ml). Cells were analyzed at a 200× magnification by fluorescence microscopy (Leica DMLB, Germany) equipped with an excitation filter of 535/50 nm, a short arc HBO 103 W/2 mercury

lamp and a barrier filter of 610/75 nm and attached to a digital camera (Applied imaging Corp., now Genetix) connected to a personal computer. Images of randomly selected cells were captured from each slide, using Cytovision (v3.0) capture software (Genetix). % DNA in tail was measured with Tritek CometScore freeware (v1.5) (www.autocomet.com). At least 50 cells were analyzed per slide (two slides per independent experience giving a total of 100 cells for each concentration per experiment) and at least three independent experiments were performed.

4.2.5 *γ-H2AX assay*

Detection of DSBs was carried out by immunofluorescence, using a FITC-antibody for phosphorylated histone H2AX (γ-H2AX). Histone H2AX is a 14 kDa ubiquitous member of the H2A histone family that contains an evolutionarily conserved SQ motif at the C-terminus in eukaryotes. Serine 139 within this motif becomes rapidly phosphorylated to yield a form known as γ-H2AX in response to double-strand DNA damage, signaling for repair and recruiting DNA repair proteins. AA8 and EM9 cells were incubated in 8 well culture Labtek II slides (Nalge Nunc International, Naperville, USA) for 24 h and then exposed to two different concentrations of eugenol and myristicin, 250 and 750 µM, during 1-h, at 37ºC, 5% CO_2. H_2O_2 (250 µM) was used as a positive control and for each sample a negative control with DMSO (0.2 % v/v) was also prepared. Cells were fixed in 4% formaldehyde in PBS for 15 min, washed with PBS and permeabilized with Triton X-100 (0.5%) at room temperature for 5 min, washed twice with 1% Bovine Serum Albumin (BSA), and then blocked with 4% BSA for 1 h. Cells were incubated with the γ-H2AX primary antibody (mouse anti- γ-H2AX (ser139), Stressgen, bioreagents corp., Canada) at 2 µg/ml for 2 h, washed twice, incubated with a FITC-conjugated goat anti–mouse second antibody (Santa Cruz Biotechnology, inc., USA) at 1 µg/ml for 1 h, washed three times more, and finally incubated with Hoechst (1 µg/ml), for 5 min and mounted with anti-fade. Cells were analyzed at a 200× amplification by fluorescence microscopy (Leica DMLB, Germany) equipped with an excitation filter of 480/40 nm, a short arc HBO 103 W/2 mercury lamp and a barrier filter at 527/30 nm and attached to a digital camera (Applied imaging Corp., now Genetix) connected to a personal computer. Images of randomly selected cells were captured from each slide, using Cytovision (v3.0) capture software (Genetix). Image analysis of γ-H2AX foci was

performed by the freeware Cellprofiler (Carpenter et al., 2006). γ-H2AX foci/nucleus were counted and integrated FITC fluorescence of γ-H2AX foci was calculated, in order to distinguish between cells with numerous foci and cells with large foci. At least two independent experiments with two replicates per experiment for each dose were performed. At least 20 nuclei were analyzed per experiment per dose.

4.2.6 *Caspases activity assay*

Caspases activity was determined using the CaspaTag™ Pan-Caspase *In Situ* Assay Kit, fluorescein (Chemicon International) as described by the manufacturer, briefly:

4.2.6.1 *Fluorimetric detection:* Twenty-four hour AA8 and EM9 cultures (approximately 1×10^6 cells), growing in 175cm^2 culture flasks at 37ºC, 5% CO_2, were exposed to 750 µM of myristicin or eugenol, after a 24 h exposure period, cells were incubated with a green Fluorochrome Inhibitors of Caspases (FLICA) (FAM-VAD-FMK) at 37ºC for 1-h. After washing, cells were counted and the density of treated and non-treated cells was compared. Treated cells were resuspended in PBS sufficient to adjust the concentration to that of the non-treated population (< 400 µL). The fluorescence was measured in a black 96-well plate using an excitation wavelength of 490 nm and an emission wavelength of 520 nm in a Zenyth 3100 microplate reader. The green fluorescent signal is a direct measure of the amount of active caspases present in the cell at the time the reagent was added. Camptothecin (CPT) (46 µM) was used as a positive control and the negative control cells were treated with DMSO (0.2% v/v).

4.2.6.2 *Fluorescence Microscopy analysis:* AA8 and EM9 cells were incubated in 8 well culture Labtek II slides (Nalge Nunc International, Naperville, USA) for 24 h at 37ºC, 5% CO_2 and then exposed to 750 µM eugenol or myristicin. After a 24 h exposure period cells were incubated with FLICA (FAM-VAD-FMK) for 1-h at 37ºC and then incubated with Hoechst (1 µg/ml) for 5 min more. After Hoechst incubation cells were washed twice with wash buffer and mounted with anti-fade (Vectashield). Cells were analyzed at a 200× and 400× magnification by fluorescence microscopy (Leica DMLB, Germany) equipped with an excitation filter of 480/40 nm, a short arc HBO 103 W/2 mercury lamp and a barrier filter at 527/30 nm attached to a personal computer. Images were captured from each slide, using Cytovision (v3.0) capture software

(Genetix). At least two independent experiments were performed, with two replicates per dose per experiment. CPT (46 µM) was used as a positive control and the negative control cells were treated with DMSO (0.2% v/v).

4.2.7 *TUNEL assay*

Apoptosis was detected by the TdT-mediated dUTP-digoxigenin nick end labeling (TUNEL) method using the ApopTag®Plus Peroxidase *In Situ* Apoptosis Detection Kit (Chemicon International, CA, USA) as described by the manufacture. Briefly, AA8 and EM9 cells grown on 8 well culture Labtek II slides (Nalge Nunc International, Naperville, USA) were treated with 250, 500 and 750 µM of myristicin or eugenol during a 24 h period at 37ºC. DMSO at 0.2% (v/v) was added to the wells without chemical. After treatment, cells were fixed in 2% formaldehyde, washed, incubated with ice-cold ethanol:acetic acid (2:1) and washed. Cells were treated with TdT enzyme at 37ºC, the reaction was stopped after 1 h and after various washes cells were incubated with anti-digoxigenin peroxidase conjugate for 30 min at room temperature followed by washing with PBS. Cells were stained with diaminobenzidin (DAB) solution, washed, counterstained with 0.5% methyl green, washed, dehydrated, and mounted. Cells were analyzed at a 400× amplification by light microscopy. To quantitate apoptosis 1000 nuclei were counted per experiment in each replicate (two replicates for each concentration were used, for each experiment). At least three independent experiments were performed.

4.2.8 *Statistical analyses*

For the Comet assay, the Shapiro-Wilk and Kolmogorov-Smirnov test were used to verify the normality of the % DNA in tail, for each slide. The results indicated no normal distribution around the median or mean, so for each batch of 50 cells from each slide the 75th percentile was calculated and used for the statistical analysis. One-way ANOVA was performed with a post-test for linear trend, as suggested by Lovell and Omori (Lovell and Omori, 2008).

For the γ-H2AX assays neither the distribution of individual foci per cell nor the integrated fluorescence per cell followed a Normal distribution, as verified by the Shapiro-Wilk and Kolmogorov-Smirnov test, and thus we opted not to compute average values. Thus, the 75th

percentile of integrated fluorescence was computed for each individual dose for each experiment. Means were computed and one-way ANOVA was performed with a post-test for linear trend. The Student's *t*-test comparing the control with individual doses was also performed to confirm the statistical analysis.

For the TUNEL assay and for the caspases assay the mean values for each experiment were calculated. Statistical analysis of the differences between mean values was carried out using the Student's *t*-test.

All statistical analyses were performed with the SPSS statistical package (version 15) (SPSS inc., Chicago, IL), and GraphPad Prism 5 (GraphPad Software, Inc).

4.3 RESULTS

4.3.1 *MTT cell viability assay*

AA8 and EM9 cells were analyzed for cell viability, after treatment with a wide range of concentrations of eugenol and myristicin, from 50 to 2000 µM for 3 h and 24 h, using the MTT assay protocol. For a 3 h exposure there were no significant reductions in cell viability, either in AA8 nor EM9 cells for eugenol (Figure 4.1 (A)), being the alkenylbenzene myristicin more cytotoxic than eugenol (73.1% viability in AA8 cells compared with 91% for eugenol for the concentration of 1000 µM) (Figure 4.2 (A)). For a 24 h exposure, the cell viability was reduced below 50% when cells were treated with concentrations higher than 500 µM and 1000 µM, for AA8 and EM9 cells, respectively (Figures 4.1 (B) and 4.2 (B)). Eugenol had 46.7% viability in AA8 cells comparing to 12.85% viability for myristicin for the concentration of 1000 µM. Therefore, for the comet assay and the γ-H2AX assay we used maximum concentrations of 500 µM and 750 µM respectively, and an incubation period of 1 h was chosen in order to minimize the influence of cell death in these assays.

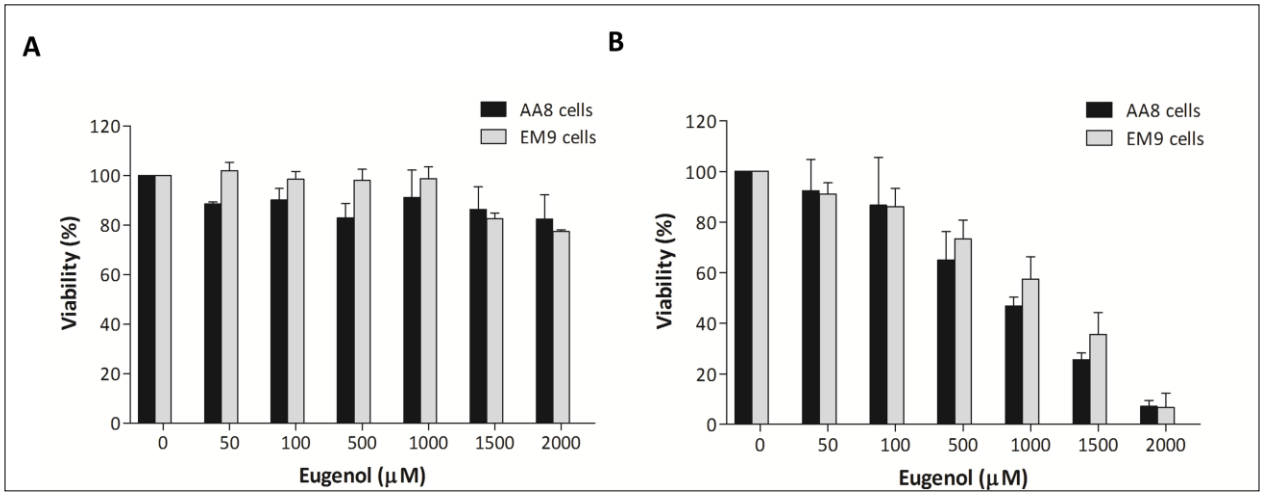


Figure 4.1 MTT viability assay in AA8 and EM9 cells after a 3 h period (A) and 24 h period (B) exposure to eugenol. Results are expressed as mean values and SE from at least two (3 h period) and three (24 h period) independent experiments.

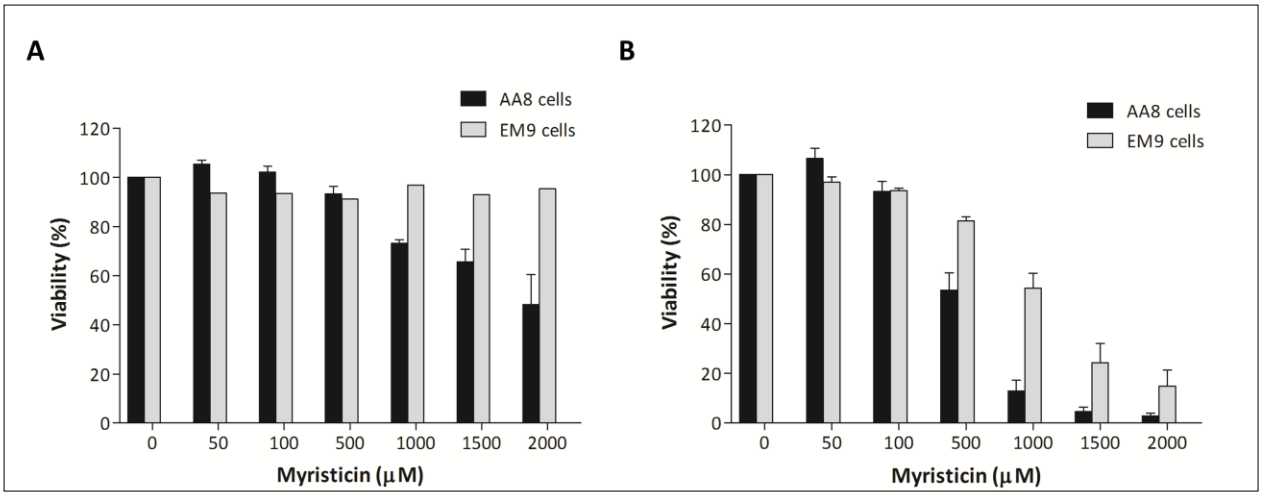


Figure 4.2 MTT viability assay in AA8 and EM9 cells after a 3 h period (A) and 24 h period (B) exposure to myristicin. Results are expressed as mean values and SE from at least two (3 h period) and three (24 h period) independent experiments.

4.3.2 *Alkaline comet assay*

The ability of eugenol and myristicin to induce DNA damage was first assessed with the alkaline comet assay. AA8 and EM9 cells were exposed to a range of concentrations from 50 to 500 µM for 1 h and the % of DNA in tail was calculated. Figure 4.4 shows the boxplots of the 75th percentiles of % DNA in tail measured after 1 h treatment for AA8 and EM9 cells. In AA8 cells,

DNA damage was induced by eugenol, but with no statistical significance (one-way ANOVA, $P = 0.0528$). However there was a significant linear trend for DNA damage ($P = 0.0132$). Myristicin did not induce DNA damage in AA8. Neither eugenol nor myristicin induced significant DNA damage in EM9 cells.

4.3.3 γ-*H2AX*

To assess if these alkenylbenzenes are also able to induce DSBs we quantified phosphorylation of H2AX at serine 139 which correlates well with each DSB (Plesca et al., 2008). Our analysis of γ-H2AX foci was performed with automatic image analysis software (CellProfiler). We analyzed both γ-H2AX foci and integrated FITC γ-H2AX fluorescence per nuclei. Integrated fluorescence per nuclei profiles was similar to of γ-H2AX foci (data not shown).

As we can see in Figure 4.4, eugenol induces significant response in DSBs after 1 h exposure period in AA8 cells (one-way ANOVA, $P = 0.0263$) and results displayed a significant linear trend ($P = 0.0097$). To confirm the analysis we also performed a Student's *t*-test comparing the control with individual doses, and eugenol showed a significant response at 750 μM in these cells ($P = 0.0255$). Myristicin did not induce significantly DSBs in AA8 or EM9 cells but in EM9 cells myristicin had a 1.9-fold increase in DSBs comparing to 1.2-fold increase in AA8 cells, as we can see in Figure 4.4, images (B) and (C). Eugenol did not displayed significant genotoxic activity in EM9 cells.

4.3.4 *Caspases activation*

Apoptotic cell death also involves activation of caspases, so we evaluated apoptosis by detection of pan activated caspases, monitored by fluorescence microscopy using a green Fluorochrome Inhibitors of Caspases assay (FLICA). The activation of caspases induced by 750 μM myristicin or eugenol, in AA8 and EM9 cells during 24 h, was analyzed. The results are shown in Figures 4.5 and 4.6. Myristicin clearly enhanced activation of caspases as evidenced by fluorimetric analysis and fluorescence microscopy (Figure 4.5 and Figure 4.6), both in AA8 and EM9 cells. There was a higher increase in EM9 cells (2.7-fold increase), relative to controls, compared to AA8 cells (1.8-fold increase). Eugenol was not able to significantly increase caspases-positive cells at the concentration of 750 μM used in either cell line.

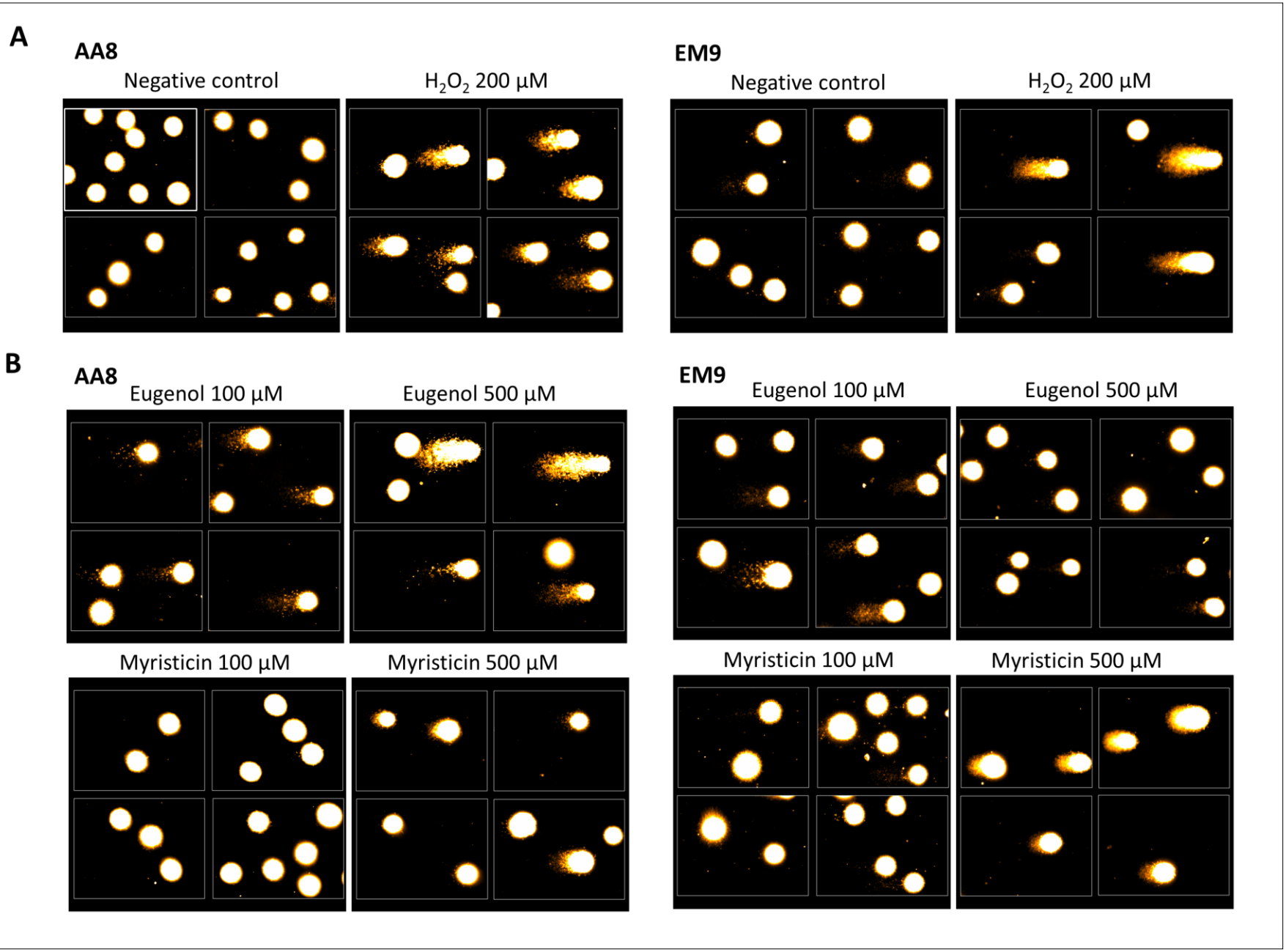

A
AA8
Negative control
H_2O_2 200 µM
EM9
Negative control
H_2O_2 200 µM
B
AA8
Eugenol 100 µM
Eugenol 500 µM
Myristicin 100 µM
Myristicin 500 µM
EM9
Eugenol 100 µM
Eugenol 500 µM
Myristicin 100 µM
Myristicin 500 µM

Figure 4.3 Detection of DNA damage induced by myristicin and eugenol in AA8 and EM9 cells using the alkaline comet assay. (A) Images show analysis of comets acquired using fluorescence microscopy of AA8 and EM9 cells. Cells exposed to DMSO were used as negative controls and hydrogen peroxide (H_2O_2 200 µM) was used as a positive control. (B) AA8 and EM9 cells after exposure to 100 and 500 µM of eugenol or myristicin.

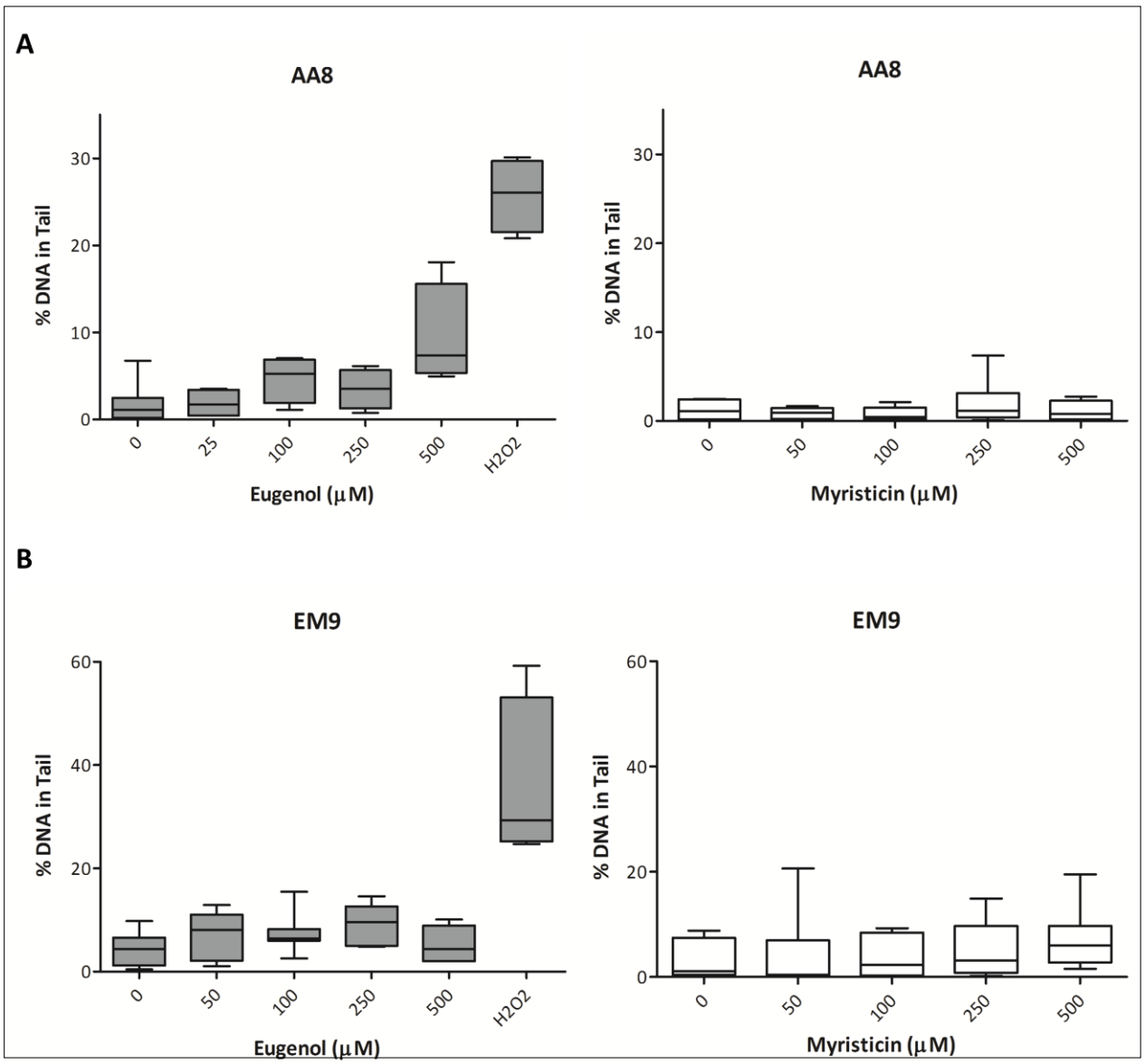
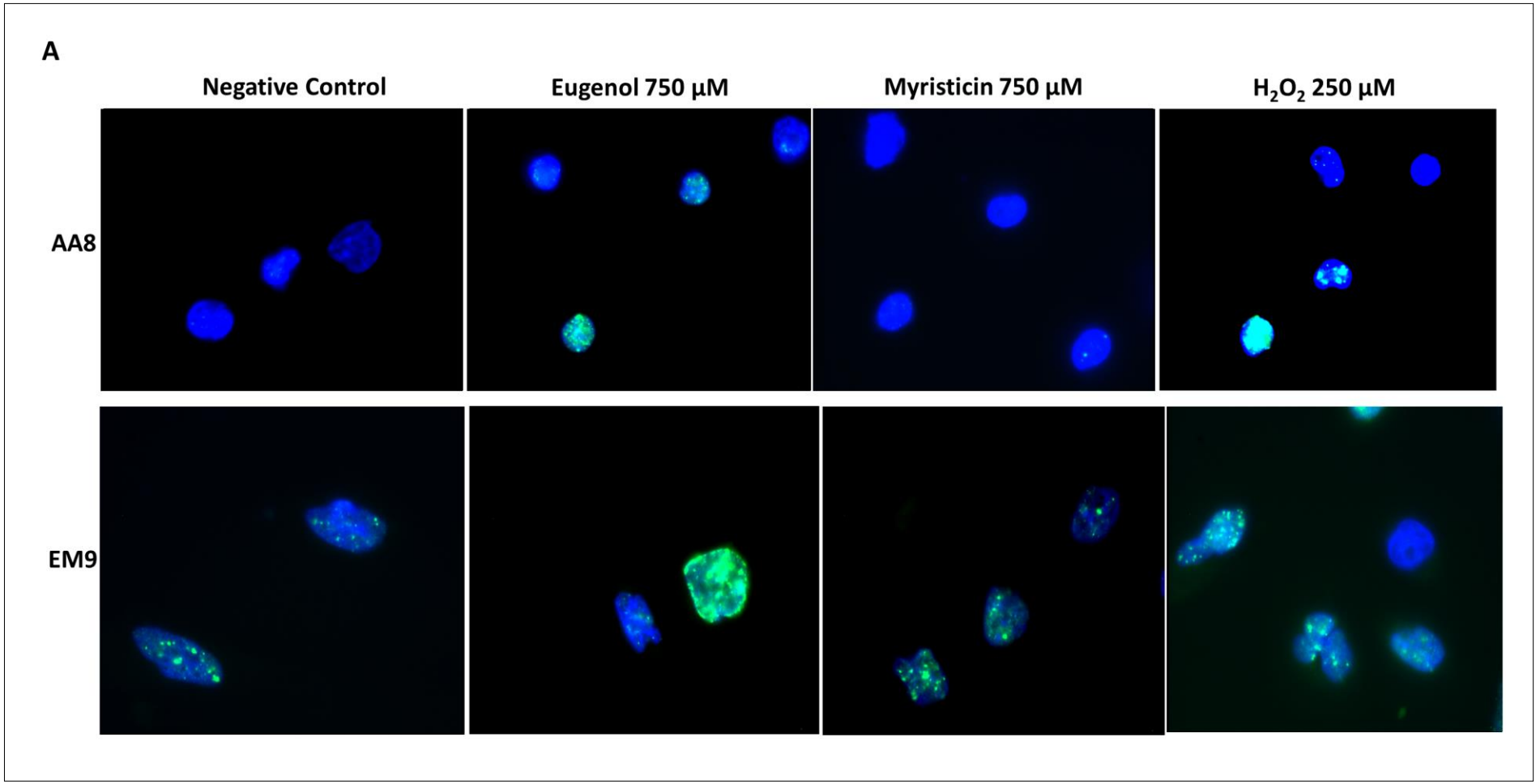


Figure 4.4 Detection of DNA damage induced by myristicin and eugenol in AA8 and EM9 cells using the alkaline comet assay. Data presented as the boxplots of 75th percentile of % DNA in Tail. Results are for treatment with concentrations between 25 to 500 µM of eugenol or myristicin during 1 h incubation period. No statistical significance (one-way ANOVA, $P = 0.0528$) was found however there was a significant linear trend for DNA damage ($P = 0.0132$) for eugenol. At least three independent experiments were performed.


A
Negative Control
Eugenol 750 µM
Myristicin 750 µM
H_2O_2 250 µM
AA8
EM9

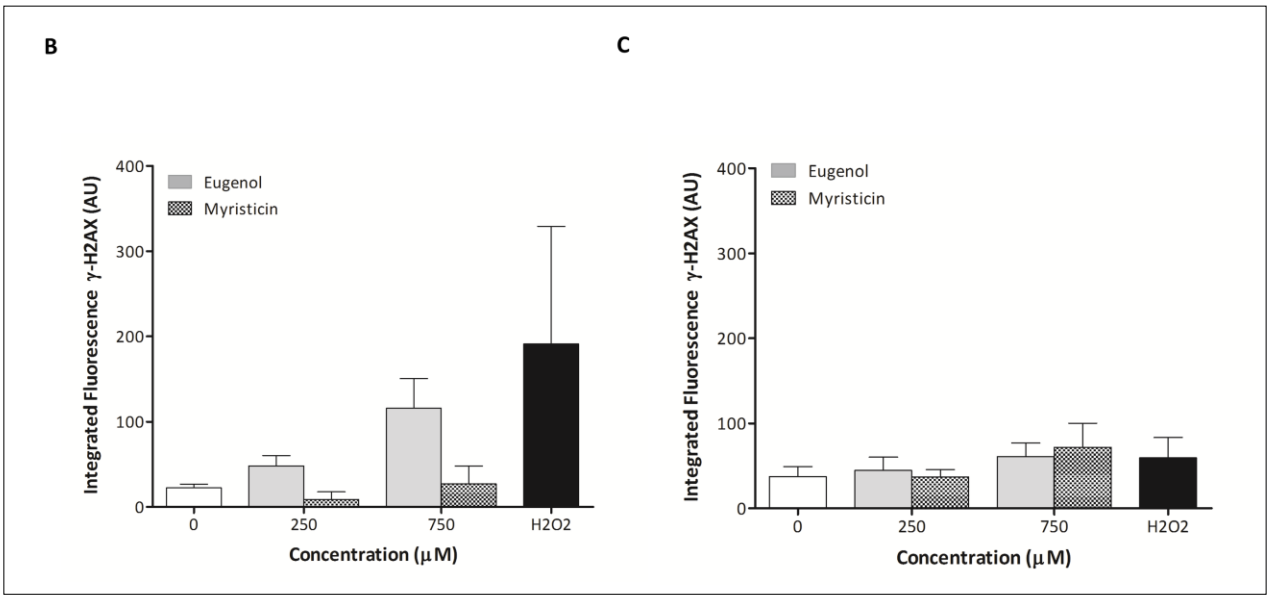
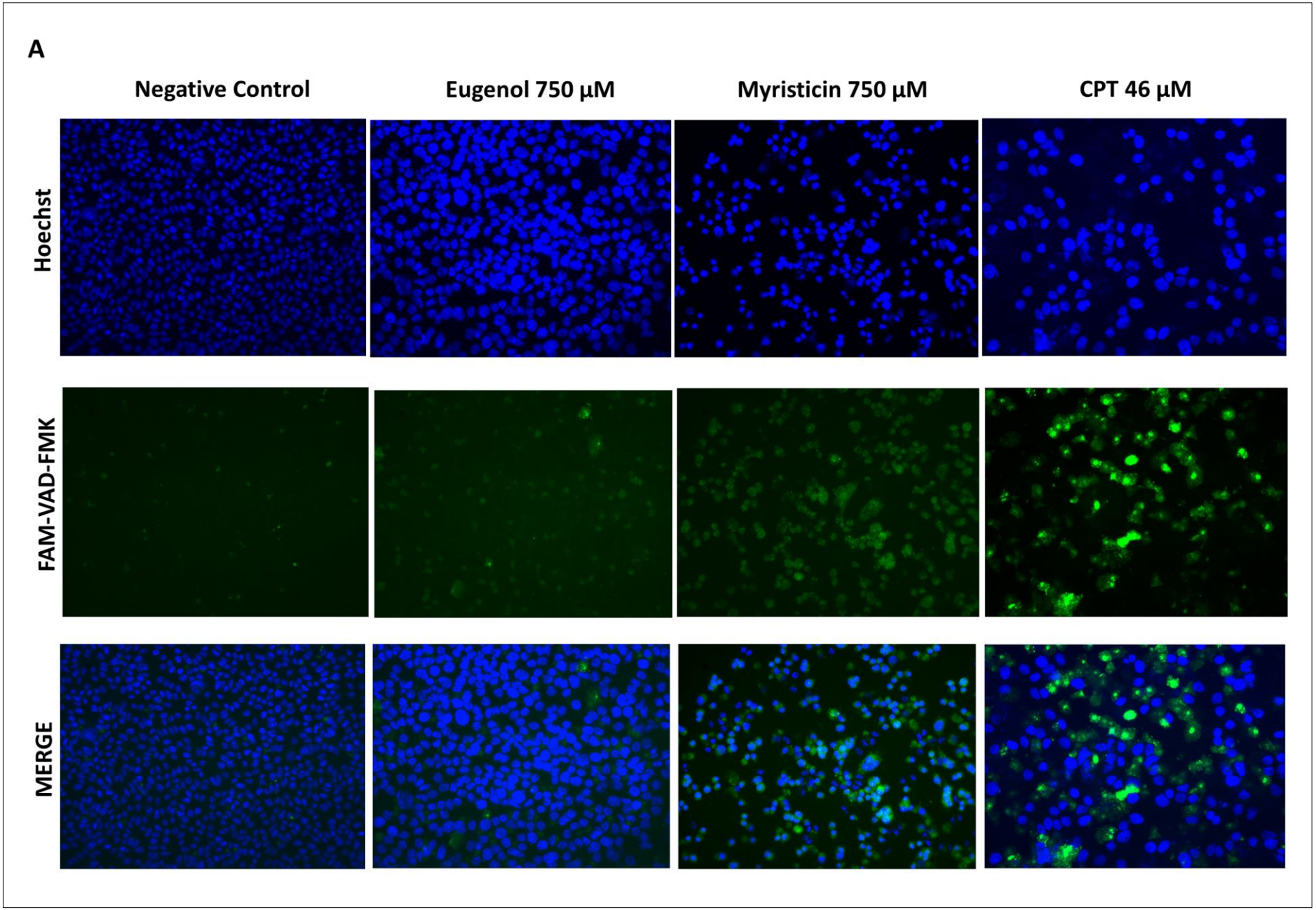


Figure 4.5 Histone H2AX phosphorylation in AA8 cells and EM9 cells treated with eugenol or myristicin. (A) γ-H2AX foci captured by fluorescence microscopy. Hydrogen peroxide (H_2O_2 250 μM) was used as positive control. (B) and (C) Bar graph values from AA8 and EM9 cells, respectively, are shown as integrated γ-H2AX fluorescence/nucleus, accessed using the freeware Cellprofiler. Results are expressed as mean values and SEM from at least two independent experiments. H_2O_2 (250 μM) was used as positive control. Eugenol induces significant response in DSBs after 1 h exposure period in AA8 cells (one-way ANOVA, *P* = 0.0263) with a significant linear trend (*P* = 0.0097).

4.3.5 TUNEL Assay

We also performed the TUNEL assay, which allows the detection of DNA fragmentation as a consequence of internucleosomal cleavage of genomic DNA, typical of cells undergoing apoptosis. TUNEL positive cells (cells shown in brown in Figure 4.7, image (A)) were observed after 24 h period exposure to 250, 500 and 750 μM of myristicin or eugenol. Almost no DNA fragmentation was observed in control cells. Quantification of cells positively labeled, showing DNA strand breaks generated during apoptosis, confirmed that myristicin induces apoptosis more efficiently than eugenol in both AA8 and EM9 cells (Figure 4.7). The percentages of TUNEL labeled cells for myristicin were 84.1 % and 69.5 % after incubation with the higher dose tested in AA8 and EM9 cells, respectively (Figure 4.7, images (B) and (C)). For eugenol the percentage of apoptotic cells was 38.9% and 35.8% in AA8 and EM9 cells, respectively. These results suggest that apoptosis can be triggered both by eugenol as by myristicin, with a higher activity for myristicin. No differences seem to exist between cell lines for both compounds.


A
Negative Control
Eugenol 750 µM
Myristicin 750 µM
CPT 46 µM
Hoechst
FAM-VAD-FMK
MERGE

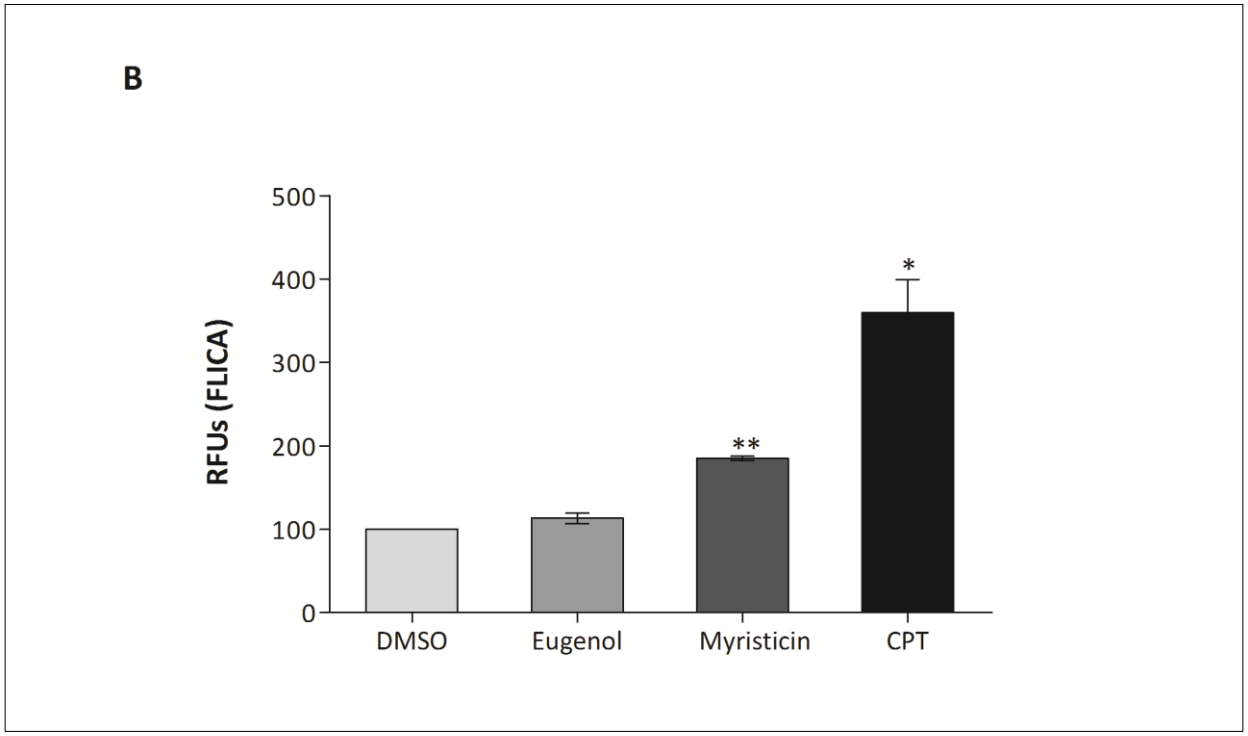
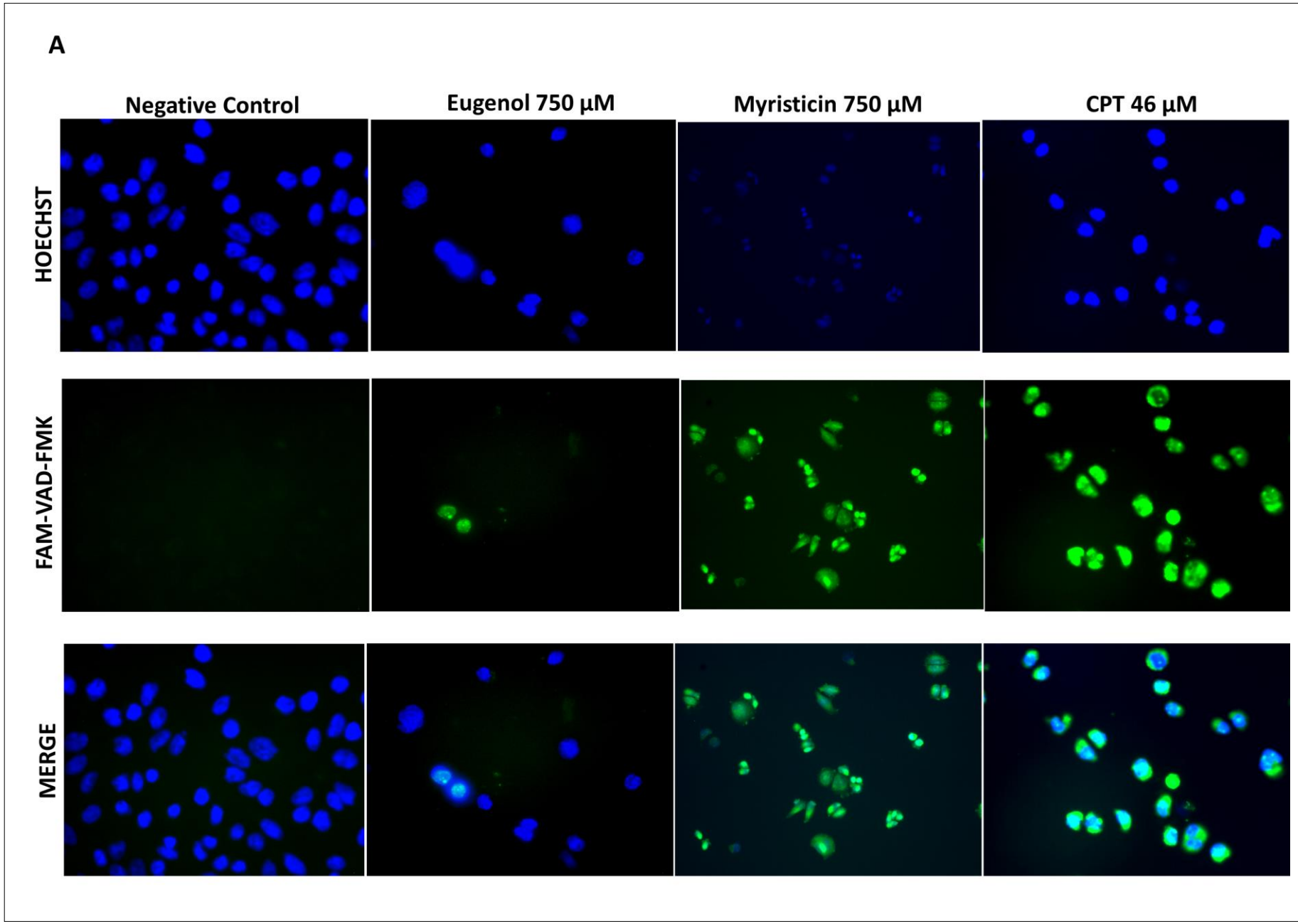


Figure 4.6 Detection of activation of caspases using FLICA in AA8 cells. Cells were treated with 750 µM of eugenol or myristicin for 24 h. Camptothecin (CPT 46 µM) was used as positive control and cells with DMSO 0.2 % (v/v) were used as negative controls. Caspases activity was determined using the CaspaTag™ Pan-Caspase *In situ* Assay Kit. (A) Images captured by fluorescence microscopy. In the first row we can see cells labeled with Hoechst stain, in the second row, cells labeled with FAM-VAD-FMK stain indicating caspase-positive cells, and in the third row we can see merge of Hoechst stain with FAM-VAD-FMK stain. (B) Activation of caspases analyzed by fluorimetric detection expressed in terms of fold increase compared to untreated cells. Values are presented as means ± SD from two independent experiments. * $P < 0.05$, ** $P < 0.001$, Student's *t*-test.


A
Negative Control
Eugenol 750 µM
Myristicin 750 µM
CPT 46 µM
HOECHST
FAM-VAD-FMK
MERGE

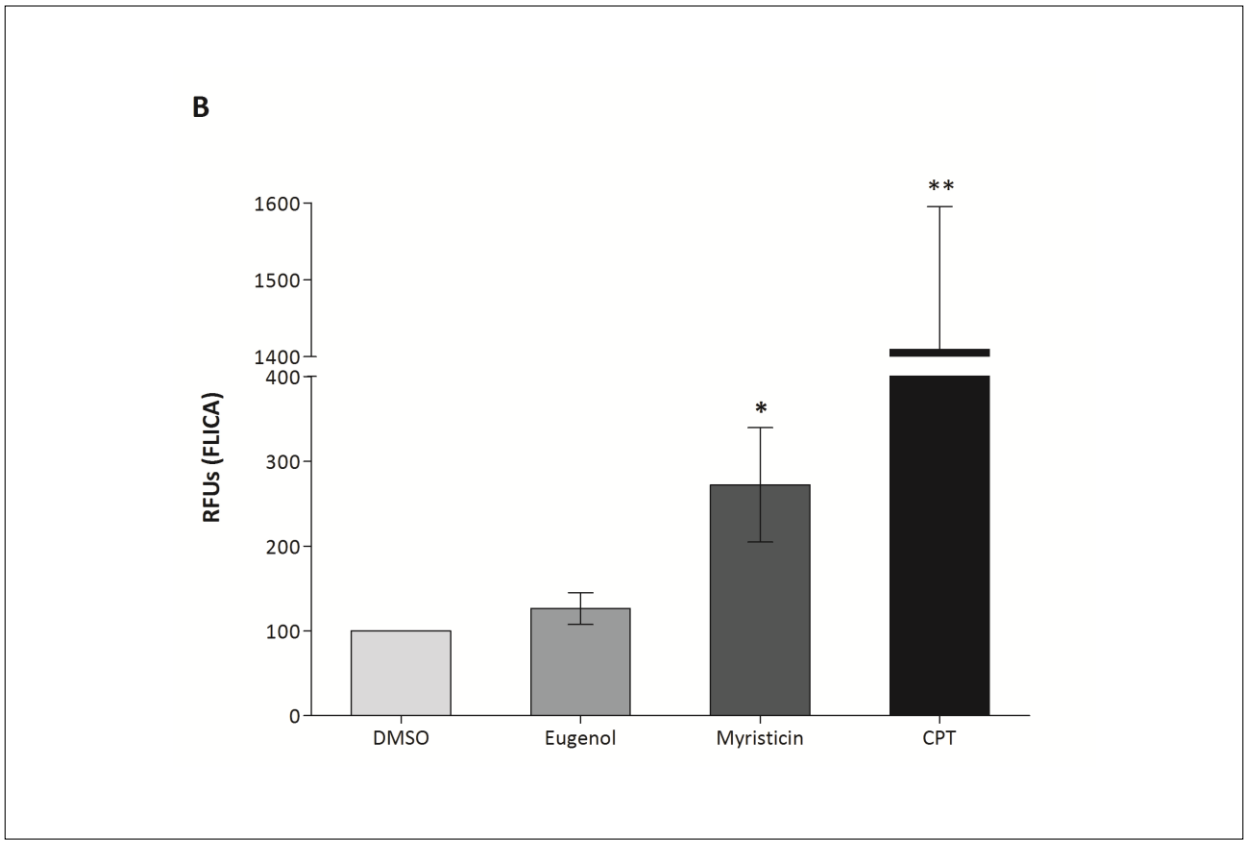


Figure 4.7 Detection of activation of caspases using FLICA in EM9 cells. Cells were treated with 750 µM of eugenol or myristicin for 24 h. Camptothecin (CPT 46 µM) was used as positive control and cells with DMSO 0.2 % (v/v) were used as negative controls. Caspases activity was determined using the CaspaTag™ Pan-Caspase *In situ* Assay Kit. (A) Images captured by fluorescence microscopy. In the first row we can see cells labeled with Hoechst stain, in the second row, cells labeled with FAM-VAD-FMK stain indicating caspase-positive cells, and in the third row we can see merge of Hoechst stain with FAM-VAD-FMK stain. (B) Activation of caspases analyzed by fluorimetric detection expressed in terms of fold increase compared to untreated cells. Values are presented as means ± SD from two independent experiments. $^{*}P < 0.05$, $^{**}P < 0.001$, Student's *t*-test.

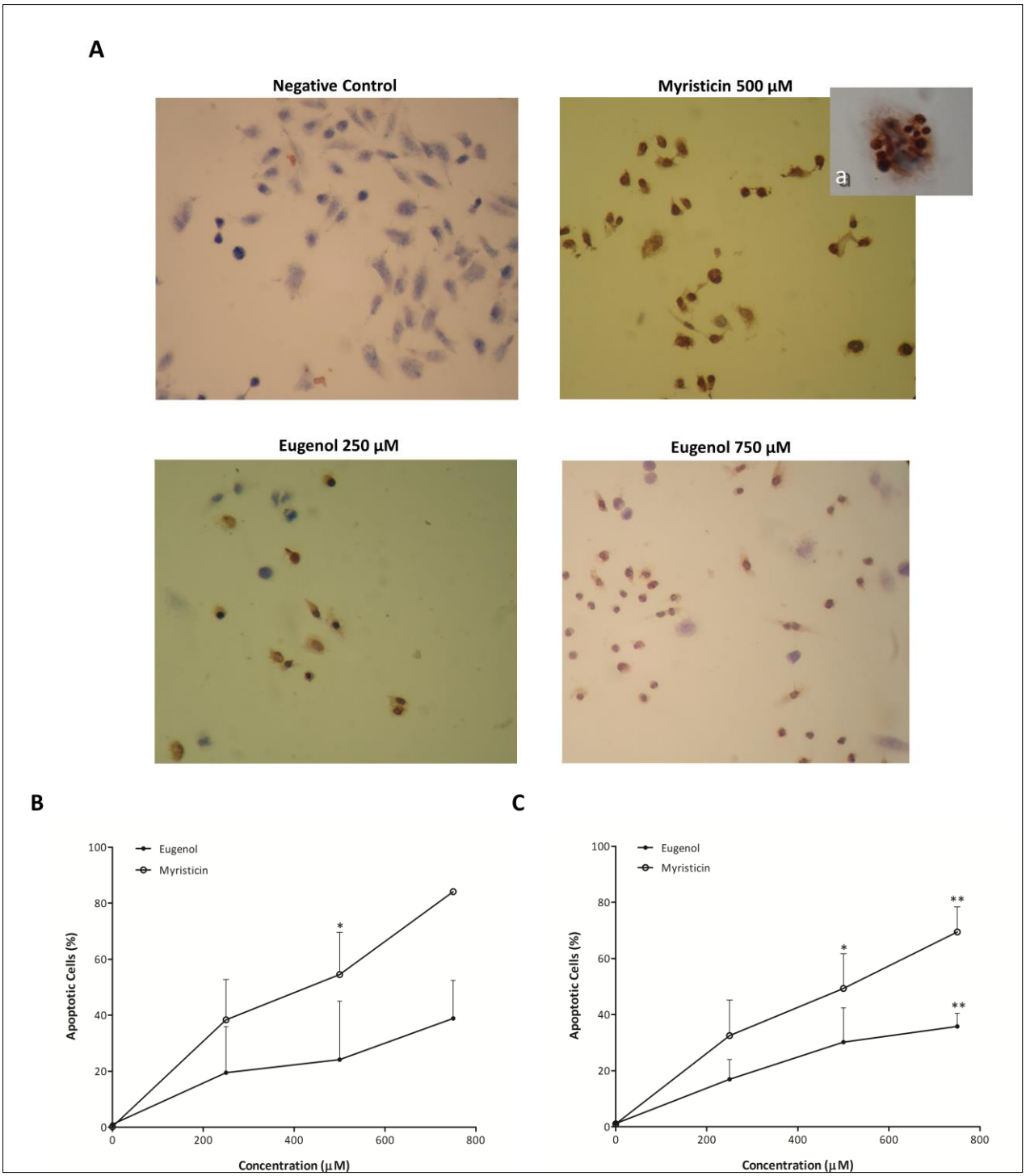


Figure 4.8 Detection of internucleossomal DNA fragmentation by the TdT-mediated dUTP-digoxigenin nick end labelling (TUNEL) in AA8 (A and B) and EM9 cells (C). TUNEL assay was performed using the ApopTag Plus Peroxidase *In situ* Apoptosis Detection Kit, cells were treated for 24 h with myristicin or eugenol. (A) Images show analysis of TUNEL positive (brown) or negative (blue) AA8 cells after exposure to 250 and 750 µM of eugenol or 500 µM of myristicin, figure (a) shows an apoptotic cell induced by myristicin. (B) Data obtained for AA8 cells expressed as means ± SE from at least three independent experiments. $^{*}P <$ 0.05, Student's *t*-test. (C) Data obtained for EM9 cells expressed as means ± SE from at least three independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$, Student's *t*-test.

4.4 DISCUSSION

Since usage of spices and flavourings is on the rise, there is concern that excessive exposure may have adverse health effects. A recent report by the Committee of experts on flavouring substances of the Council of Europe (Coe, 2006) reported an average consumption of about 9 mg/day of myristicin from spices, and about 4 mg/day from essential oils. These values rise to 32 mg/day from spices and 14 mg/day from essential oils if considering an extreme intake of a 97.5th percentile consumer. Very rarely voluntary intoxications due to nutmeg ingestion (5-15 g) have occurred, in order to experience hallucinogenic effects, with a clinical course of shock, coma and acidosis (Barceloux, 2008). For eugenol, estimates of intake vary from about 1 mg/day in the European Union to about 3 mg/day in the US (EFSA, 2009). These values are not of immediate concern but increased consumption of spices and teas containing these flavourings could merit attention. In addition, combined dietary exposures of different flavourings with structural similarities should also be taken into account when assessing risk (JECFA, 2008).

Safrole is classified as a carcinogen, through a mechanism involving DNA adduct formation, yet screening of other alkenylbenzenes for adduct formation showed equivalent total adduct levels formed by myristicin *in vitro* (Zhou et al., 2007), raising the possibility that myristicin does have indeed DNA binding activity and is possibly carcinogenic. However, safrole binding to DNA *in vivo* exceeds binding by myristicin by a factor of 3-4 (Randerath et al., 1993). On the other hand, myristicin did not induce UDS in hepatocytes derived from male Fischer 344 rats in doses up to 10 mM, ten times the dose where cytotoxicity was noted (Hasheminejad and Caldwell, 1994). Thus there is uncertainty on the *in vivo* effects of myristicin but a suggested DNA binding mechanism for myristicin. The differences observed between safrole and myristicin could indicate differences in DNA repair of lesions, which could involve nucleotide excision repair (NER) but also BER. Indeed, (Phillips et al., 1984), using 32P-postlabeling, observed that in comparison to the carcinogenic compounds estragole, methyleugenol and safrole, adducts of myristicin were less persistent. If myristicin does display DNA binding activity, the general lack of genotoxicity and carcinogenicity is interesting. (Auerbach et al., 2010), using a toxicogenomics and machine learning approach to predict the hepatocarcinogenicity of alkenylbenzene flavouring agents, including eugenol and myristicin, reached the conclusion that myristicin would be

hepatocarcinogenic if studied at a dose level of 2 mmol/kg bw/day for 2 years in male F344 rats, thus making myristicin a higher priority relative to other untested alkenylbenzenes for evaluation in the carcinogenicity bioassay.

The results presented here indicate that eugenol and myristicin display differences in genotoxicity and apoptosis in Chinese hamster ovary cells (CHO). In AA8 cells, after 1 h incubation, eugenol induces DNA strand breaks as measured by the comet assay and DSBs as measured by the γ-H2AX assay.

In chapter II we showed that eugenol induces CAs and endoreduplication in V79 cells suggesting a genotoxic mechanism, possibly as a topoisomerase II inhibitor. CAs formation can arise due to DSBs and/or misrepair of DSBs (Natarajan and Palitti, 2008) and DSBs can be induced directly (i.e. ROS) or indirectly during the repair process which can convert single strand lesions into DSBs (Nakamura et al., 2003; Natarajan and Palitti, 2008). Irrespectively of the type of DNA lesions induced, ultimately they have to be converted to DSBs in order to give rise to CAs (Natarajan and Palitti, 2008). Our positive results with the

γ-H2AX assay for eugenol are in accordance with our previous results with the CA assay (chapter II), showing that eugenol can induce DSBs.

In the XRCC1 repair deficient EM9 cells, however, eugenol was not genotoxic, although we could expect a higher genotoxicity in EM9 cells compared to repair-proficient AA8 cells. EM9 cells are known for their high sensibility to alkylating agents and ionizing radiation (Thompson et al., 1982). However when challenged with hydrogen peroxide, we did not observe a much higher genotoxicity in EM9 cells compared to AA8 cells (median value for AA8 cells 26 % of DNA in tail *vs* 29% in EM9 cells). These results are consistent with observations by other authors (Cantoni et al., 1987; Stearns et al., 2005). Cantoni et al. (Cantoni et al., 1987) showed that EM9 cells display a reduced ability to remove oxidative lesions from their DNA, due to the absence of XRCC1. Additionally, (Wong et al., 2005) showed that the primary biochemical defect associated

with XRCC1 deficiency in EM9 cells was in the ligation step of BER/SSB repair, after processing of oxidative lesions, being 2-4-fold lower than in AA8 cells, thus leading to a several-fold reduction in the repair of SSBs. Taken together, these results indicate that EM9 cells are relatively refractory to the genotoxic effects of oxidative agents, contrary to AA8 cells. The comet assay measures

essentially SSBs which are frequently induced by these DNA repair processes, whereby oxidative lesions are processed by the BER pathway (Nakamura et al., 2003). DSBs are not considered to contribute greatly to the damage detected in the comet assay, due to their rarity (Caldecott, 2003), although they can be formed when two SSBs arise in close proximity (Jackson and Bartek, 2009), being detected by the γ-H2AX assay. Thus our results can be grounded if we consider that the genotoxicity of eugenol is partly due to oxidative lesions and SSB due to the BER pathway. Eugenol has been shown to produce ROS which can cause oxidative damage and SSBs and induce chromosome breakage (Kligerman et al., 2010). In AA8 cells this pathway is active, whereas in EM9 cells, due to a deficiency in XRCC1, this pathway does not give rise to SSBs or DSBs. In contrast, myristicin was not genotoxic in either AA8 or EM9 cells, either in the comet assay or the γ-H2AX assay.

Both eugenol and myristicin induce apoptosis after a 24 h incubation period, yet myristicin showed to be more potent than eugenol (Figures 4.5 and 4.6). One of the earliest and most consistent observed features of apoptosis is the activation of caspases, a well-known biochemical marker of apoptosis (Plesca et al., 2008). Caspases are synthesized as inactive precursors, which are activated by proteolytic cleavage to generate active enzymes. Our results show that myristicin activated caspases in both AA8 and EM9 cells, although presenting a higher caspase activating activity in EM9 cells, indicating therefore that the apoptotic activity of myristicin is caspase-dependent. Conversely, eugenol did not activate caspases. Internucleosomal cleavage of DNA measured by the TUNEL assay belongs to the late features of apoptosis. Our results with eugenol are in agreement with (Yoo et al., 2005), who observed that eugenol induced DNA fragmentation and mitochondrial permeability transition in HL-60 cells via production of ROS. These results are also in agreement with (Lee et al., 2005), who showed that myristicin induces cytotoxicity in human neuroblastoma SK-N-SH cells by an apoptotic mechanism. The direct mechanisms involved in the induction of apoptosis by myristicin are not known. Interestingly, the apoptotic activity of these flavourings does not seem to correlate with their genotoxic activities, since eugenol is more genotoxic than myristicin, yet myristicin displays higher apoptotic activity than eugenol.

Taken together, our results indicate that the XRCC1 protein does not seem to be essential for the repair of lesions induced by eugenol and does not influence its apoptotic activity.

Our results may contribute to a further understanding of the potential risk of increasing our consumption of these alkenylbenzenes present in the diet, as was recommended by the JECFA meeting in 2008 (JECFA, 2008).

4.5 REFERENCES

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5. Chapter

MYRISTICIN FROM NUTMEG INDUCES APOPTOSIS VIA THE MITOCHONDRIAL PATHWAY AND DOWN REGULATES GENES OF THE DNA DAMAGE RESPONSE PATHWAYS IN HUMAN LEUKAEMIA K562 CELLS

This chapter was adapted from:

Myristicin from nutmeg induces apoptosis via the mitochondrial pathway and down regulates genes of the DNA damage response pathways in human leukaemia K562 cells

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ABSTRACT

Myristicin, an alkenylbenzene, is a major active component of various spices, such as nutmeg and cinnamon, plants from the *Umbelliferae* family or in some essential oils, such as oils of clove or marjoram. Human exposure to myristicin is low but widespread due to consumption of these spices and essential oils, added to food (e.g. cola drinks) or in traditional medicine. Occasionally high dose exposure occurs, leading to various clinical symptoms, however the molecular mechanisms underlying them are unknown.

Our previous studies revealed that myristicin is not genotoxic and yet presented apoptotic activity. Therefore, in this work we assessed the apoptotic mechanisms induced by myristicin in human leukaemia cells. In order to gain further insight on the potential of myristicin to modulate gene expression we also analysed alterations in expression of 84 genes associated with the DNA damage response pathway. The results obtained show that myristicin can induce apoptosis as characterized by alterations in the mitochondrial membrane potential, cytochrome c release, caspase-3 activation, PARP-cleavage and DNA fragmentation. The gene expression profile revealed an overall down regulation of DNA damage response genes after exposure to myristicin, with significant under-expression of genes associated with nucleotide excision repair (ERCC1), double strand break repair (RAD50, RAD51) and DNA damage signalling (ATM) and stress response (GADD54A, GADD45G). On the whole, we demonstrate that myristicin has no-genotoxicity measured by the comet assay and the γ-H2AX assay, but can alter mitochondrial membrane function, induce apoptosis and modulate gene expression in human leukaemia K562 cells. This study provides further detail on the molecular mechanisms underlying the biological activity of myristicin.

5.1 INTRODUCTION

Alkenylbenzenes such as myristicin are present as natural constituents of various spices and food flavours and in some botanical families (e.g. *Apiaceae, Lamiaceae, Myrtaceae* and *Rutaceae*) (Bakkali et al., 2008; Miller et al., 1983). Myristicin is the major component of the aromatic ether fraction of the essential oil of mace, and nutmeg aromatic fraction contains essentially myristicin, elemicin and safrole that account for 85-95% of the compounds, while myristicin represents about 4-12% of the compounds present in the essential oil (Barceloux, 2008).

Human exposure to myristicin is widespread due to consumption of spices, such as nutmeg, basil, anise, cinnamon, clove, fennel, star anise, plants from the *Umbelliferae* family including dill, celery, parsnip, parsley, and carrot or in some essential oils, such as oils of clove, marjoram, bay leaf and cinnamon leaf (Hallstrom and Thuvander, 1997). Nutmeg oil is a major constituent of cola flavourings. Thus myristicin is present in cola drinks (mean 168.3 µg/L), together with another alkenylbenzene safrole (mean 23 µg/L) (Raffo et al., 2013). There has been a steady increase in the consumption of cola drinks worldwide. Additionally, certain prepared foods, such as puddings, sweet sauces, and baked goods, contain nutmeg at a level that can reach 0.3% by weight (Randerath et al., 1993). Furthermore, nutmeg, mace and dill preparations are also used in traditional medicine (e.g. Chinese, Indonesian, Ayurveda) to treat rheumatism, cholera, psychosis, stomach cramps, nausea, diarrhoea, and anxiety (Barceloux, 2008; Jana and Shekhawat, 2010; Ozaki et al., 1989). A report by the Committee of experts on flavouring substances of the Council of Europe (Coe, 2006) reported an average consumption of about 9 mg/day of myristicin from spices, and about 4 mg/day from essential oils. However, exposure to myristicin due to traditional medicine is not known. The consumption of natural compounds as phytochemicals in teas or in functional foods to enhance health is increasing in interest, as are their uses in CAM (Aggarwal et al., 2008; Anand et al., 2008; Bakkali et al., 2008; Rietjens et al., 2008). Research indicates that 40% of US consumers reported using herbal products, representing a 25% increase in a 7-year period (Leiter et al., 2011). Furthermore, many people believe CAM are safe (or carry no risk of harm). However, traditional medicines and practices can cause adverse reactions if the product or therapy is of poor quality, or is taken inappropriately or in conjunction with other drugs (Zuba and Byrska, 2012). Additionally, intentional ingestion of

high doses of nutmeg, containing myristicin, has occurred in users trying to obtain a “nutmeg high”, reaching up to 21 g/person, leading to blood levels of 2 mg/mL (Stein et al., 2001). In these cases, symptoms predominantly involve the central nervous and cardiovascular systems (Demetriades et al., 2005), however the molecular mechanisms underlying these symptoms are not known. Fatal cases of nutmeg poisoning have been reported, with exposures leading to twice as high blood levels as described above (Stein et al., 2001).

For alkenylbenzenes, data obtained thus far has focused essentially on their genotoxic and carcinogenic activities (Maralhas et al., 2006; Martins et al., 2012; Martins et al., 2011). Hence, these dietary components should be more thoroughly studied to improve risk evaluation, and more information on other biological activities is needed. The JECFA considered that a mechanistic understanding of the toxic effects of alkoxy-substituted alkenylbenzenes, such as myristicin, and estragole, and their implications for human risk have yet to be fully explored, and will have a significant impact on the assessment of health risks at the concentrations at which they occur in food, thus requiring more information on their biological effects (JECFA, 2008).

In the past few years, several studies have focused on the activity of non-nutritional dietary compounds that have protective or disease preventive properties. These phytochemicals include carotenoids, food polyphenols, such as flavonoids, phytoalexins, phenolic acids indoles and sulphur rich compounds and compounds found in spices, such as curcumin, genistein and resveratrol (D'Incalci et al., 2005; Sporn and Suh, 2002; Surh, 2003). Some of them are already in clinical trials to ascertain their potential use in cancer chemoprevention (Amin et al., 2009). Due to the variety of their physiological roles in plants, some phytochemicals have been associated with pleiotropic effects in animal cells (D'Incalci et al., 2005; Neergheen et al., 2010; Russo, 2007; Shu et al., 2010; Sporn and Suh, 2002; Surh, 2003; Thomasset et al., 2007). They can alter cell cycle control, apoptosis and cell survival pathways, cell migration and metastasis. Thus, in contrast to small-molecule pharmaceuticals designed for acting at defined targets, dietary phytochemicals affect a large number of cellular targets with varied affinities that, in combination, result in recognized health benefits (Arango et al., 2013).

In our previous chapters we demonstrated that myristicin is not genotoxic but displays apoptotic activity, mediated by activation of caspases, in hamster ovary CHO cells (Martins et al., 2011).

In this chapter we analysed genotoxicity in K562 cells with the comet assay and the γ-H2AX assay and evaluate the mechanisms of apoptosis and cell survival in human chronic myeloid leukaemia K562 cells after exposure to myristicin. Additionally, to gain insight on the potential of myristicin to deregulate signalling pathways, we also analysed the gene expression profile of 84 genes associated with the DNA damage response pathway, using real-time PCR.

5.2 MATERIAL AND METHODS

5.2.1 *Chemicals and Reagents*

Myristicin (CAS No. 607-91-0), MTT and digitonin as all the other reagents, unless otherwise specified, were obtained from Sigma–Aldrich (St. Louis, MO, USA). DMSO, ethanol, sodium chloride, potassium chloride, magnesium chloride and acid acetic were obtained from Merck KGaA (Darmstadt, Germany). Formaldehyde solution (16% w/v) was obtained from Thermo scientific (Rockford, USA). Nonidet P-40 from USB Corporation (Cleveland, OH, USA) and the protease inhibitor cocktail from Roche Diagnostics, Mannheim, Germany.

5.2.2 *Cell Culture*

Human leukaemia K562 cells (DSMZ, Germany) were routinely maintained in 175 cm^2 culture flasks in RPMI supplemented with 10% foetal bovine serum and 1% antibiotic solution (penicillin–streptomycin) at 37ºC, under an atmosphere containing 5% CO_2.

5.2.3 *MTT viability assay*

Approximately 1-5 × 10^4 cells were cultured in complete medium growing in 96-well plates pre-treated with poly D-lysine (Millipore, USA) as described by the manufacture. The cells were allowed to grow for 24 h and then exposed to different concentrations of myristicin (dissolved in DMSO, not exceeding 0.2 %), ranging from 50 to 1000 µM, for 24, 48 and 72 h periods. DMSO at 0.2 % (v/v) was added to the wells without chemical (control cultures). At the end of the treatment, the medium was removed and the MTT, dissolved in culture medium, was added to

each well at a concentration of 0.5 mg/ml. Cells were grown for a further period of 3 h and then the media was discarded and the DMSO (200 µl) added to each well to dissolve the formazan crystals. Absorbance was read at 595 nm in a Zenyth 3100 microplate reader. Absorbance values presented by control cultures correspond to 100 % cell viability. At least three independent experiments were performed.

5.2.4 *Alkaline comet assay*

The comet assay was performed under alkaline conditions essentially as described by Singh et al. (Singh et al., 1988) and in chapter IV. Cells were incubated with concentrations of myristicin, ranging from 500 to 750 µM, for 1 h and for a longer period of 72 h. Cells with DMSO were used as negative control, and 200 µM of Hydrogen Peroxide (H_2O_2) was used as a positive control. Images of randomly selected cells were captured from each slide, using Cytovision (v3.0) capture software (Genetix). % of DNA in tail was measured with Tritek CometScore freeware (v1.5) (www.autocomet.com). At least 50 cells were analyzed per slide (two slides per independent experience giving a total of 100 cells for each concentration per experiment) and two or four independent experiments were performed for 1 h (500 and 750 µM of myristicin) and 72 h (750 µM of myristicin), respectively.

5.2.5 *γ-H2AX assay*

Detection of DSBs was carried out by immunofluorescence, using a FITC-antibody for phosphorylated histone H2AX (γ-H2AX) as described in chapter IV. K562 cells were incubated in 8 well culture Labtek II slides (Nalge Nunc International, Naperville, USA) for 24 h and then exposed to 250, 500 or 750 µM of myristicin during 1 h. H_2O_2 (250 µM) was used as a positive control and for each sample a negative control with DMSO (0.2 % v/v) was also prepared. Cells were analyzed at a 200× amplification by fluorescence microscopy (Leica DMLB, Germany) equipped with an excitation filter of 480/40 nm, a short arc HBO 103 W/2 mercury lamp and a barrier filter at 527/30 nm and attached to a digital camera (Applied imaging Corp., now Genetix) connected to a personal computer. Images of randomly selected cells were captured from each slide, using Cytovision (v3.0) capture software (Genetix). Image analysis of γ-H2AX foci was

performed by the freeware Cellprofiler (Carpenter et al., 2006). γ-H2AX foci/nucleus were counted and integrated FITC fluorescence of γ-H2AX foci was calculated, in order to distinguish between cells with numerous foci and cells with large foci. At least 20 nuclei were analyzed per experiment per dose.

5.2.6 *JC-1 assay*

The mitochondrial membrane potential (ΔΨm) was analysed by staining the cells with the cationic mitochondrial dye 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine iodide, JC-1 (Molecular Probes; Eugene, OR, USA). In cells with intact ΔΨm, JC-1 dye accumulates into the mitochondrial matrix, forming JC-1 aggregates and emitting red fluorescence. In cells with collapsed ΔΨm, JC-1 remains in a monomeric form in the cytoplasm emitting green fluorescence, decreasing red fluorescence. To assess JC-1 aggregates, an equal number of K562 cells (5×10^4) were cultured for 24 h on a 96-well plate, and then exposed to 1.25 µM JC-1 for 15 min at 37ºC. After washing, the cells were exposed to myristicin in stain-free Dulbecco's phosphate buffered saline (without calcium chloride and magnesium chloride) at a concentrations range of 10 - 200 µM. Carbonyl cyanide 3-chlorophenylhydrazone (CCCP) at 0.05 mM was used as positive control. CCCP causes an uncoupling of the proton gradient that is established during the normal activity of electron carriers in the mitochondrial electron transport chain. The fluorescence was measured with a microplate reader Zenyth 3100 (Ex 485 nm; Em JC-1-Red 595 nm) after 20 min of incubation with myristicin. At least two independent experiments were performed.

5.2.7 *Caspase-3 activity assay*

Caspases-3 activity was determined using the Caspase-3 Colorimetric Assay Kit (R&D Systems, Minneapolis, MN, USA) as described by the manufacturer, briefly: Twenty-four hour K562 cultures were exposed to 250 and 500 µM of myristicin and after a 24, 48 or 72 h exposure periods, cells were washed with PBS and collected in lysis buffer on ice for 10 min. The lysates were centrifuged at 10,000g at 4°C for 1 min. Cell lysates (1×10^6 cells = 4 - 6 mg/mL protein) were incubated with caspase-3 specific substrate (DEVD-pNA) with reaction buffer in a 96-well

plate at 37°C for 1 h. The caspases activity was determined by measuring the Optical Density at 405 nm (Zenyth 3100 microplate reader) corresponding to the released p-nitroaniline and results were expressed as fold increase in caspases activity of apoptotic cells over that of non-induced cells. Camptothecin (CPT) (0.1, 5 and 10 µM during a 24, 48 or 72 h exposure period, respectively) was used as a positive control and non-induced cells were treated with DMSO (0.2 % v/v). At least two independent experiments were performed.

5.2.8 *Western blot analysis*

5.2.8.1 *Preparation of total cell lysates:* The method was performed essentially as described by Bhushan et al. (Bhushan et al., 2007) with minor modifications. Briefly, after cells were treated with 100, 250 and 500 µM of myristicin or 5µM of CPT during a 48 and 72-h periods. Cells were washed in ice-cold PBS and incubated with cold lysis buffer (50 mM Tris pH 8.0, 150 mM NaCl, 5 mM EDTA, 1% Nonidet P-40 (v/v), 1 mM PMSF and 1% protease inhibitor cocktail (v/v)) at final concentrations of 40×10^6/ml. After 30 min on ice cells were centrifuged at 12,000 ×g for 10 min at 4ºC and the supernatant was collected as whole cell lysates.

5.2.8.2 *Preparation of cytosolic and mitochondrial lysates:* The method was performed essentially as described by Maianski et al. (Maianski et al., 2004) with minor modifications. Briefly, to obtain subcellular fractions, cells were treated with 100 and 250 µM of myristicin during 6 and 12 h periods, then washed in ice-cold PBS and resuspended in the ice-cold cytosol extraction buffer (250 mM sucrose, 70 mM KCl, 250 µg/ml digitonin, 2 mM PMSF, 1% protease inhibitor cocktail (v/v) in PBS) at final concentrations of ~23×10^6/ml. After 15 min incubation on ice, the preparations were spun at 1,000×g for 5 min, and the supernatants were kept as cytosolic fractions. The pellets were resuspended in the same volume (as the cytosol extraction buffer) with the ice-cold mitochondria lysis buffer (100 mM NaCl, 10 mM $MgCl_2.6H_2O$, 2 mM EGTA, 2 mM EDTA, 1% Nonidet P-40 (v/v), 10% glycerol (v/v), 2 mM PMSF, 1% protease inhibitor cocktail (v/v) in 50 mM Tris, pH 7.5) and incubated for 10 min on ice, followed by a 10-min centrifugation at 10,000×g. The supernatants were collected as mitochondrial fractions.

5.2.8.3 *Protein quantification:* Protein was measured using the Bradford Method (Bio-Rad Protein Assay; Bio-Rad laboratories, München, Germany), according to the manufacturer's microassay procedure.

5.2.8.4 *Immunoblotting technique:* Western blot was performed using Mini-Protean®TGX™ Precast gels (Bio-Rad, Hercules, CA, USA), according to the manufacturer's procedure. Each well was loaded with 40-60 µg of total and cytosolic proteins and 20 µg of mitochondrial proteins. Proteins were then transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore, USA). Protein detection was performed using primary antibodies anti-PARP (1:1000) and anti-β-actin (1:1000) from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-cytochrome c (1:500) from BD Pharmingen™ (BD Biosciences) and anti-COX IV (1:2000) from Abcam (Abcam, Cambridge, UK). PVDF membranes were treated with the WesternDot™ 625 Western blot kit (Invitrogen Molecular Probes, Eugene, USA), according to the manufacturer's procedure. Finally, membranes were irradiated with UV light (Fluo-Link transilluminator; Vilber Lourmat), and images were captured using Fuji film 55,000 with a 55 mm UV Jessop filter. Each Western blot was performed in at least two independent assays.

5.2.9 *TUNEL assay*

Apoptosis was detected by the TdT-mediated dUTP-digoxigenin nick end labelling (TUNEL) method using the ApopTag®Plus Peroxidase *In Situ* Apoptosis Detection Kit (Chemicon International, CA, USA) as described by the manufacture. Briefly, 24-h K562 culture cells, grown on poly-D-lysine coated microslides (Santa Cruz), were treated with a range of myristicin concentrations from 50 to 750 µM during 48-h and 72-h (data not shown). DMSO at 0.2% (v/v) was added to the wells without chemical and Imatinib (0.5 µM, Toronto Research Chemicals, Inc.) was used as positive control. After treatment, cells were fixed in 2% formaldehyde solution, washed, incubated with ice-cold ethanol:acetic acid (2:1) and washed. Cells were treated with TdT enzyme at 37ºC, the reaction was stopped after 1 h and after various washes cells were incubated with anti-digoxigenin peroxidase conjugate for 30 min at room temperature followed by washing with PBS. Cells were stained with diaminobenzidin (DAB) solution, washed, counterstained with 0.5% methyl green, washed, dehydrated, and mounted. Cells were analysed

at 400 × amplification, by light microscopy. To quantitate apoptosis 1000 nuclei were counted per experiment in each replicate (two replicates for each concentration were used, for each experiment). At least two independent experiments were performed.

5.2.10 *Real-time PCR*

To analyse alterations in gene expression we used the Human DNA Damage Signalling RT^2 Profiler™ PCR Array (Qiagen, SA Biosciences). This array profiles the expression of 84 genes involved in the DNA damage-response pathways, namely apoptosis, cell cycle and DNA Repair, together with five housekeeping genes. Controls for genomic DNA contamination and for the efficiency of the RT-PCR reaction are also provided by the assay. Total RNA (1 µg) was extracted from K562 cells without and with exposure to 100 µM myristicin for a 6 h exposure period (AllPrep DNA/RNA/Protein MiniKit, Qiagen). After assessing RNA yield and quality, cDNA was synthesized with the RT² First-Strand cDNA Synthesis Kit (Qiagen, SA Biosciences). PCR was performed on the 7300 RT PCR systems (Applied Biosystems) and the results were analysed with RT ^{2} PCR Data Analysis Software (Qiagen) using the comparative CT method. Three independent experiments were performed. All assays were performed according to protocols from the manufacturer. To further confirm our results we analysed gene expression of selected genes by individual assays. Total RNA was converted to cDNA using the High Capacity RNA-to-cDNA kit (Applied Biosystems) according to the manufacturer's instructions. Expression was determined using Taqman assays (Taqman®Universal Master Mix II) from Applied Biosystems (details of each gene are in Table 5.1). Relative gene expression level was normalized with GADPH (4352934E; Applied Biosystems). Differences between K562 cells exposed to myristicin (Test) and non-exposed (Control) cells are presented as fold change based on calculation of $2^{-\Delta\Delta Ct}$. RT-PCR was performed in triplicate for each selected gene and repeated in three independent experiments.

5.2.11 *Statistical analyses*

The results of the MTT test are represented as mean ± SD (standard deviation). To obtain the IC50 from the results of the MTT assay, non-linear regression analysis was applied. For the TUNEL assay mean values were compared through one-way analysis of variance (ANOVA). For the

caspases assay the mean values for each experiment were calculated. Statistical analysis of the differences between mean values was carried out using the Student *t*-test. All statistical analyses were performed with the GraphPad Prism 5 software (GraphPad Software, Inc.).

Table 5.1 Taqman gene expression ID (Applied Biosystems) for target genes analyzed.

Genes	Assay ID
ATM	Hs01112355_g1
ERCC1	Hs01012158_m1
GADD45α	Hs00169255_m1
GADD45γ	Hs00198672_m1
MRE11A	Hs00967443_m1
RAD 50	Hs00990023_m1
RAD 51	Hs00153418:m1
XRCC3	Hs00193725_m1

5.3 RESULTS

5.3.1 *MTT viability assay*

We first examined the effect of myristicin on the viability of K562 cells by the MTT assay. Cells were treated with a wide range of myristicin concentrations, from 50 to 1000 µM for 24, 48 and 72 h. Myristicin displayed similar dose-dependent decreases in viability as we can see in Figure 5.1 for both 24 and 48 h periods. Compared to the control, cell viability dropped >20% after exposure to 500 µM of myristicin for both 24 and 48 h exposure periods. For a 72 h exposure, myristicin significantly reduced cell viability in a concentration-dependent manner, which was reduced below 50 % when cells were treated with concentrations higher than 250 µM (IC50 = 368 µM; R^2 = 0.95).

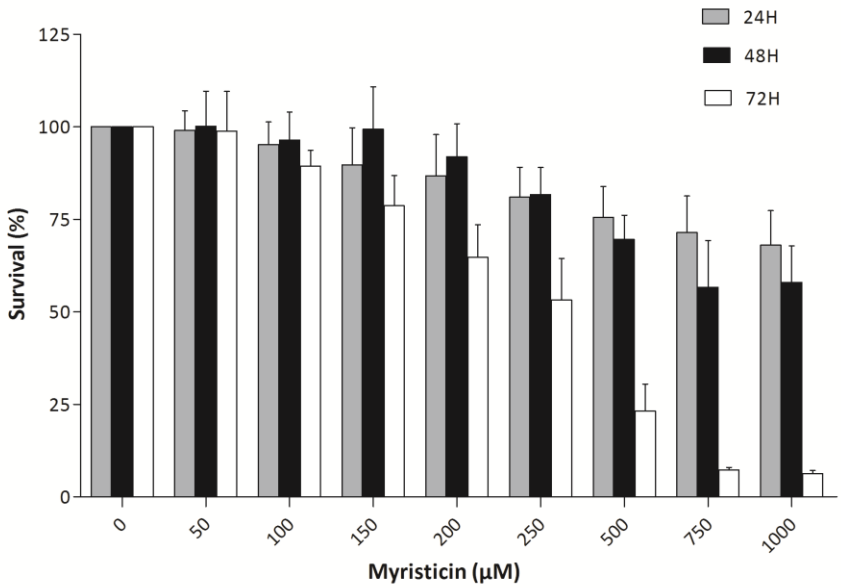
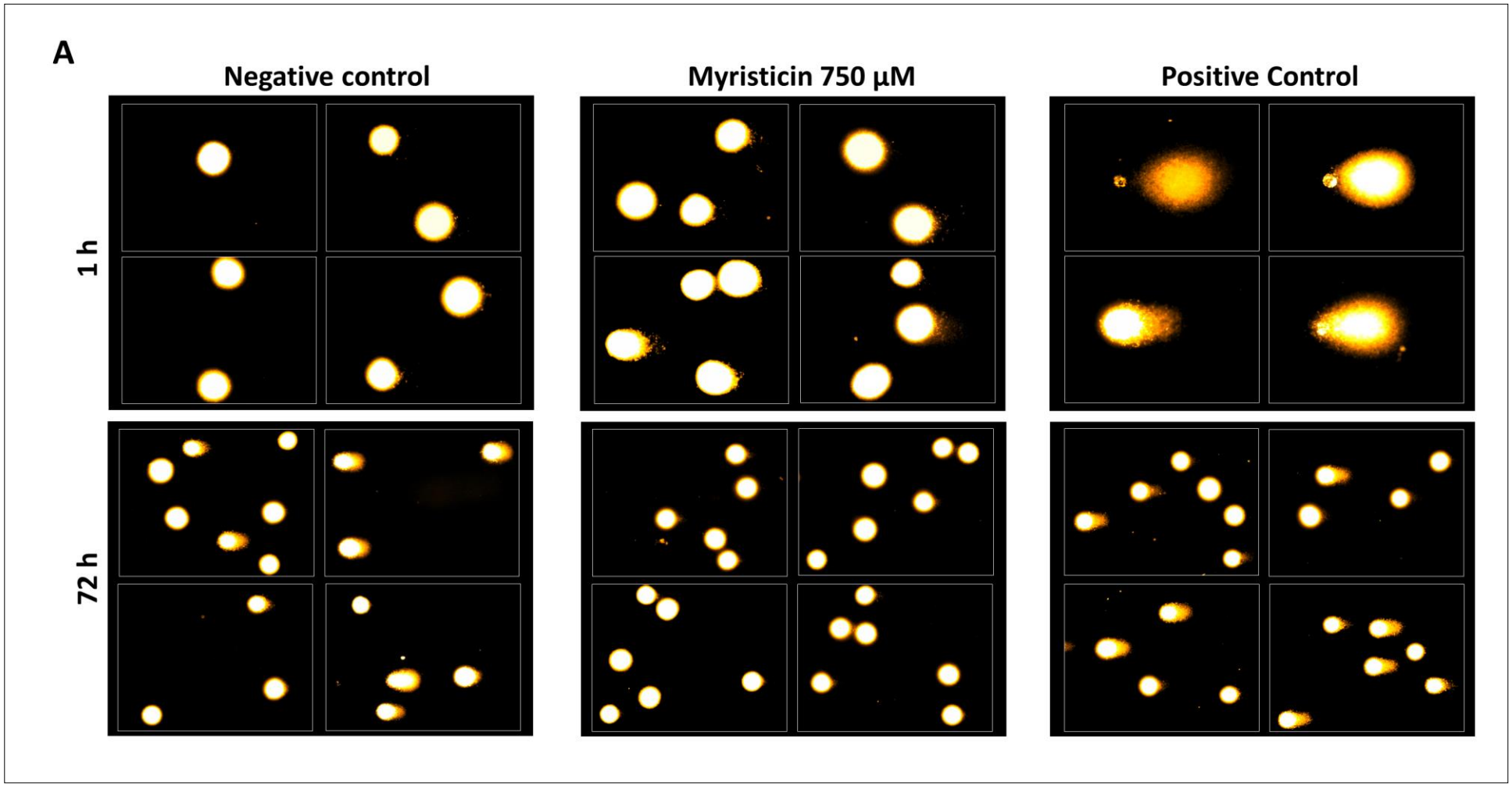


Figure 5.1 MTT viability assay in K562 cells after 24, 48 and 72 h of exposure to myristicin. Data are expressed as mean ± SD.

5.3.2 *Genotoxicity assays*

The ability of myristicin to induce DNA damage in human leukemia cells K562 was first assessed with the alkaline comet assay. Cells were exposed to 500 and 750 µM of myristicin for 1 h and 72 h and the % of DNA in tail was calculated. Figure 5.2 A) shows images captured by fluorescence microscopy, and there is no visible response for myristicin. Data from Figure 5.2 B) shows the % of DNA in tail measured after 1 h and 72 h treatments, myristicin did not induce DNA damage in either period.

Next we assessed if myristicin was able to induce DSBs, for that we quantified phosphorylation of H2AX at serine 139 (Plesca et al., 2008). Our analysis of γ-H2AX foci was performed with automatic image analysis software (CellProfiler). We analyzed both γ-H2AX foci and integrated FITC γ-H2AX fluorescence per nuclei. γ-H2AX foci were similar to integrated fluorescence per nuclei profiles (Figure 5.3 B and C, respectively). As we can see in Figure 5.3, myristicin did not induced significantly DSBs in these cells, but we can relate positive γ-H2AX cells with apoptosis as signaled with the blue arrow (Figure 5.3 A, enhanced in the black and white image (a)).


A
Negative control
Myristicin 750 µM
Positive Control
1 h
72 h

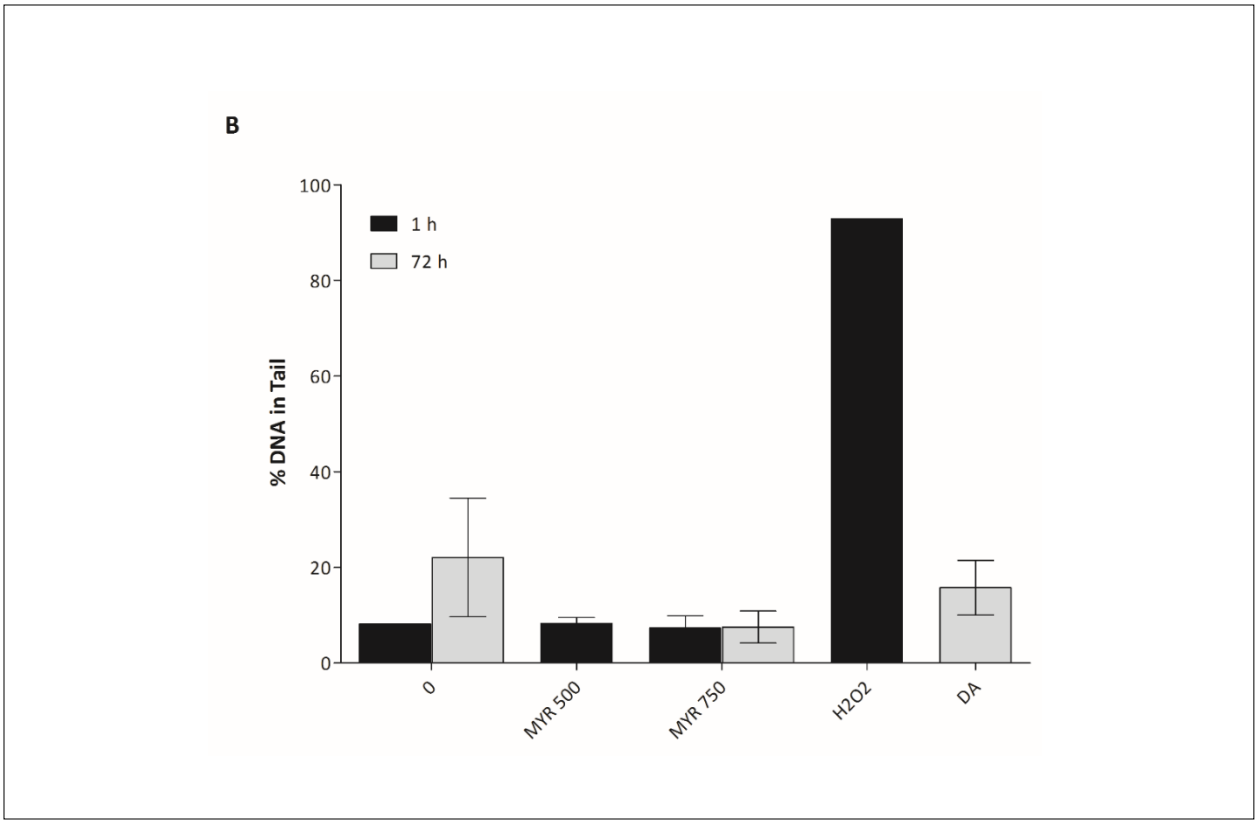
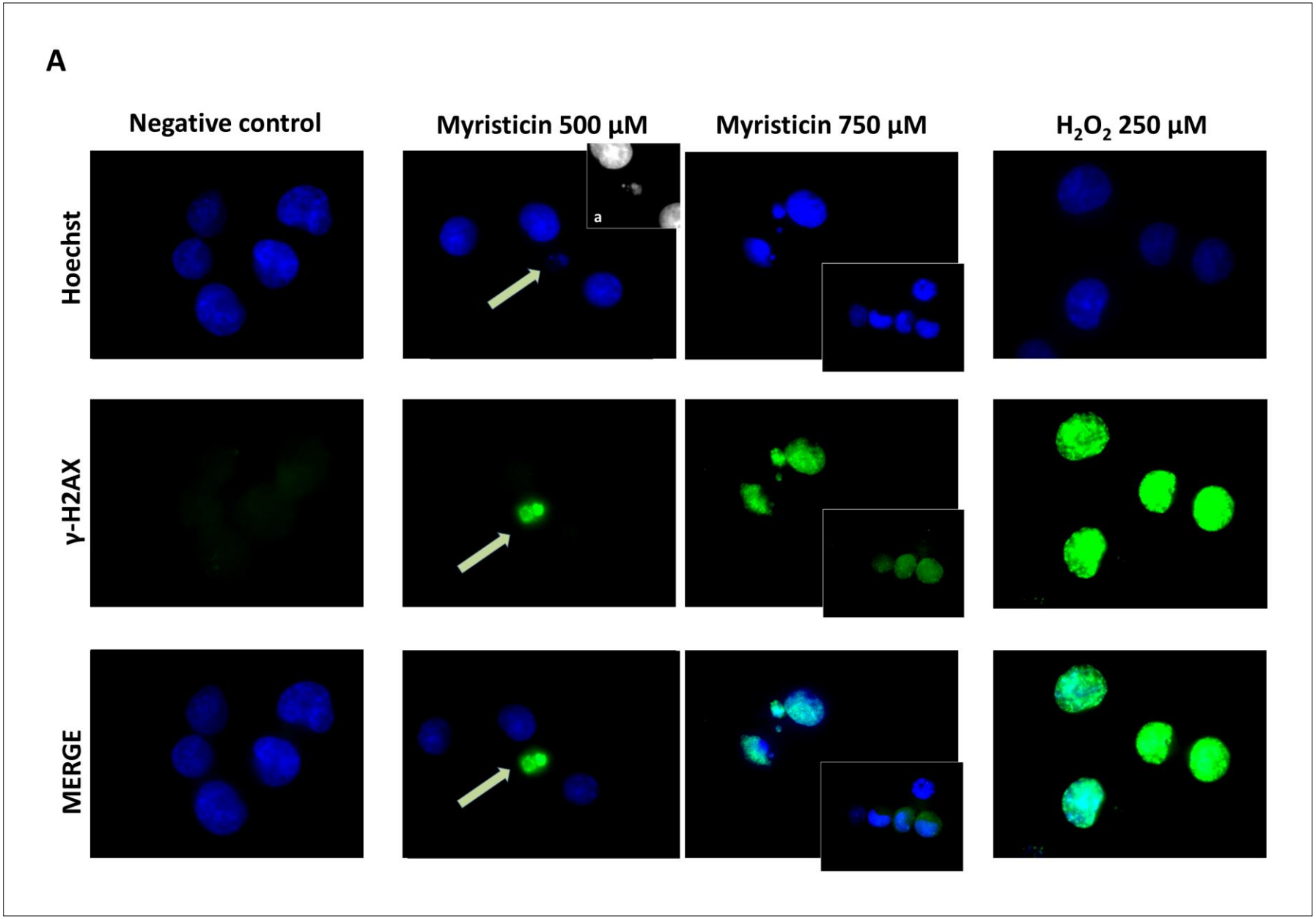


Figure 5.2 Detection of DNA damage induced by myristicin in K562 cells using the alkaline comet assay. (A) Images show analysis of comets acquired using fluorescence microscopy. Cells exposed to DMSO were used as negative controls and hydrogen peroxide (H_2O_2 200 µM) or Desatinib (DA 1.5nM) were used as positive controls for 1 h and 72 h exposure periods, respectively. (B) Data presented as mean ± SD of % DNA in Tail. Results are for treatment with concentrations of 500 and 750 µM of myristicin during 1 h and 72 h incubation periods. No statistical significance was found. At least two independent experiments were performed, the concentration of 500 µM was not performed for the period of 72h.


A
Negative control
Myristicin 500 µM
Myristicin 750 µM
H2O2 250 µM
Hoechst
γ-H2AX
MERGE
a

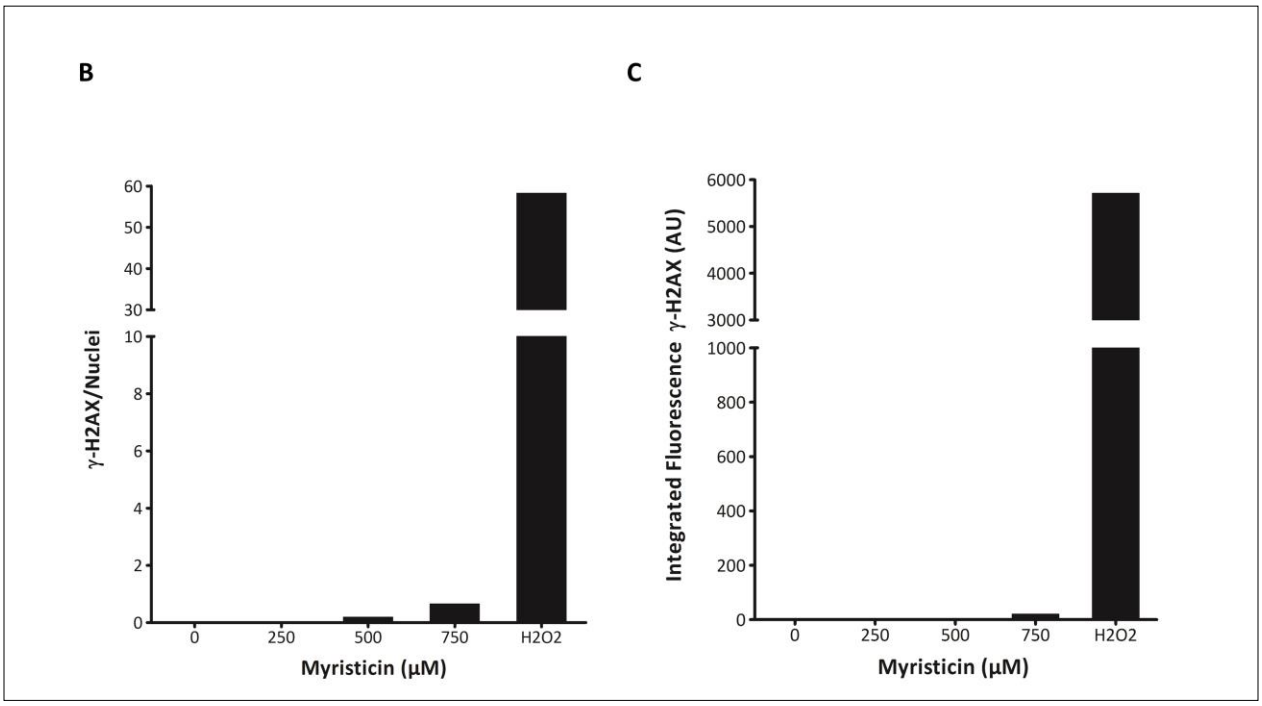


Figure 5.3 Histone H2AX phosphorylation in K562 cells treated with myristicin. (A) γ-H2AX foci captured by fluorescence microscopy; a) evidence of an apoptotic cell (sign with an arrow). Hydrogen peroxide (H_2O_2 250 μM) was used as positive control. In the first row we can see cells labeled with Hoechst stain, in the second row, cells labeled with γ-H2AX antibody indicating green positive cells, and in the third row we can see merge of Hoechst stain with γ-H2AX. Graph data are shown as γ-H2AX/nuclei (B) and integrated intensity of γ-H2AX fluorescence × γ-H2AX/nuclei (C), accessed using the freeware Cellprofiler. Results are expressed as mean values of fluorescence positive cells from images captured from at least 20 nuclei per experiment per dose. H_2O_2 (250 μM) was used as positive control. No significant response was induced by myristicin after 1 h exposure period.

5.3.3 *JC-1 assay*

To evaluate the mechanisms of apoptosis in K562 cells, we first investigated if myristicin could perturb the mitochondrial transmembrane potential. Although dissipation of the mitochondrial transmembrane potential may not be an absolute requirement for apoptosis, it can be a feature of the event that allows proteins such as cytochrome c to efflux into the cytosol (Kleinberg and Davidson, 2009). We used the membrane-permeable cationic fluorochrome JC-1. Thus, we stained K562 cells with JC-1, and measured JC-1 aggregates (red fluorescence) using a microplate reader. After a 20 min incubation period with myristicin, K562 cells decreased the red fluorescence starting from 50 μM in a dose-dependent manner as shown in Figure 5.4, indicating loss of mitochondrial membrane integrity, suggesting that mitochondrial dysfunction is induced

by myristicin. Red fluorescence decreased 12% and 39% for the lowest (50 µM) and highest (200 µM) concentration tested, respectively. CCCP was added as positive control because CCCP causes a quick mitochondrial membrane depolarization. CCCP, as we can see in Figure 5.4, caused a 65% reduction in the red fluorescence.

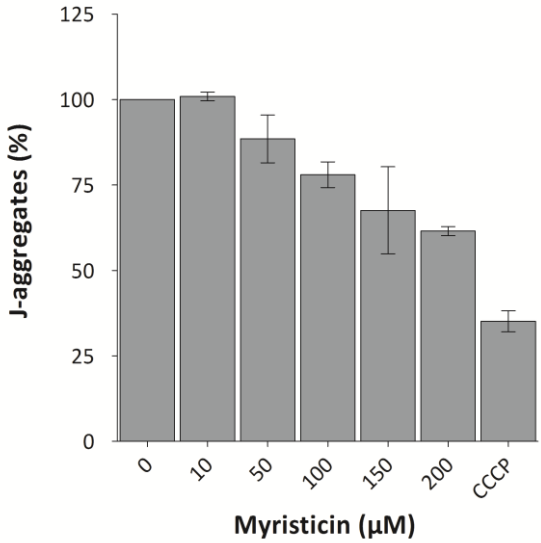


Figure 5.4 JC-1 red fluorescence decrease in K562 cells. K562 cells were stained with JC-1 (1.25 µM) and analysed in a microplate reader. Mitochondrial membrane integrity was accessed after 20 min of exposure to myristicin. CCCP was used as positive control. Data are expressed as mean ± SD of % decrease relatively to control. Absorbance values presented by control cultures correspond to 100% red fluorescence.

5.3.4 *Myristicin causes cytochrome c release from mitochondria*

Loss of mitochondrial membrane integrity during apoptosis can in turn induce the release of pro-apoptotic factors from mitochondria, e.g. cytochrome-c (Ulivieri, 2010). To determine if myristicin induces release of cytochrome-c in K562 cells after the early mitochondrial disruption observed, we treated the cells with concentrations of 100 and 250 µM using two incubation periods of 6 and 12 h and evaluated release of cytochrome c by Western blot analysis. Myristicin induced a concentration-dependent release of mitochondrial cytochrome c into the cytosol after a 6-h incubation period, indicating that the apoptotic pathway is mediated by mitochondria (Figure 5.5). After 12 h we observed a reduction of cytochrome c in the cytosol, suggesting a recovery of the mitochondrial membrane integrity.

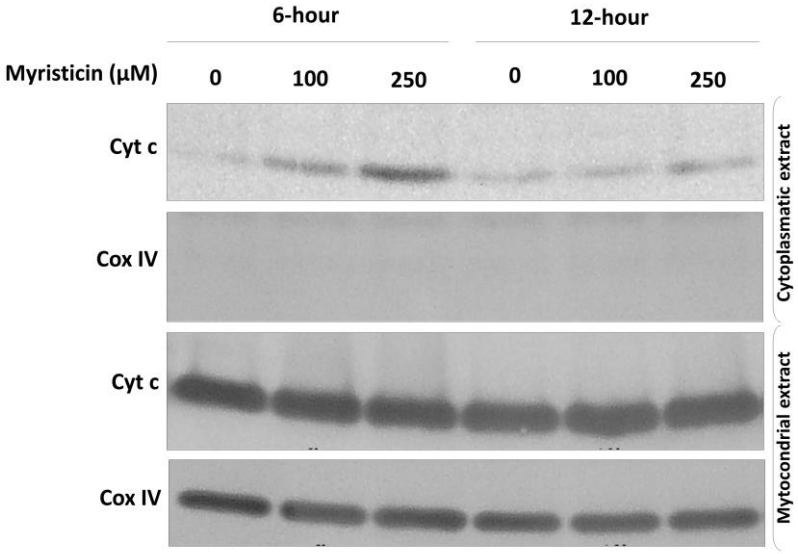


Figure 5.5 Release of cytochrome c from mitochondria to the cytosol in K562 cells. Cells were harvested at 6 and 12 h periods after incubation with 100 and 250 μM of myristicin. For the cytoplasmatic extract we used an equal amount of 60 μg protein and for the mitochondrial extract we used an equal amount of 20 μg protein. The cytosol and mitochondrial fractions were immuno-blotted with the monoclonal antibody for cytochrome c. The COXIV antibody was used as an internal control.

5.3.5 *Caspase-3 activation*

Following cytochrome c release to the cytosol it subsequently binds to Apaf-1 which results in the formation of a complex known as the apoptosome. The apoptosome can recruit procaspase-9 and thereby activate it through oligomerisation which then leads to the activation of executioner caspase-3, -6 and -7 common to both the intrinsic and extrinsic pathway (Jin and El-Deiry, 2005; Portt et al., 2011). Additional examination of this pathway was performed to determine whether caspase-3, which plays an essential role as an executor of apoptosis, might be activated during the induction of apoptosis by myristicin in K562 cells. We measured caspases-3 activity spectrophotometrically after DEVD-*p*NA cleavage (colour molecular reporter pNA) in K562 lysates collected at 24, 48 and 72 h after treatments with 250 and 500 μM myristicin. As shown in Figure 5.6, caspase-3 activity increased in a time and concentration-dependent manner, after exposure to 250 and 500 μM being statistically significant for 48-h ($P \leq 0.005$) and 72 h ($P < 0.01$). These results indicate that myristicin induces apoptosis in K562 cells through activation of caspase-3.

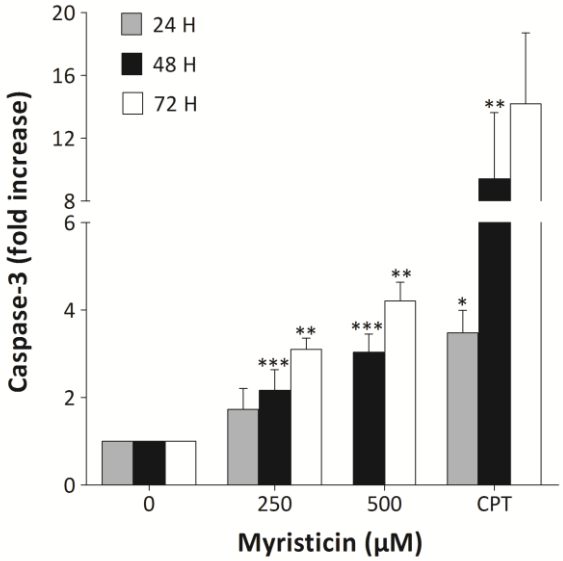


Figure 5.6 Effect of myristicin on caspases-3 activity. Caspase-3 activity was detected by DEVD-pNA at 24, 48 and 72 h after treatment with 250 and 500 μM of myristicin. Data are expressed as mean ± SD of fold increase. The *y* axis represents the fold increase comparing to control (cells without myristicin). $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.005$.

5.3.6 *PARP cleavage*

Mitochondrial pro-apoptotic effectors induce massive activation of caspases that in turn activate a proteolytic cascade leading to degradation of cellular components. Effector caspases are responsible of the cleavage of target proteins, including PARP (Jin and El-Deiry, 2005; Ulivieri, 2010)**.** Thus, since PARP is known to be a major substrate for active caspase-3 and a hallmark of apoptosis, Western blot analysis was performed to analyse expression of PARP in K562 cells with 100, 250 and 500 μM of myristicin for a 48 and 72-h periods (Figure 5.7). After a 48 h exposure the ~85 kDa cleavage product of PARP increased up to a concentration of 250 μM, with a decrease in the intensity of the band for the highest concentration tested of 500 μM. For the 72 h exposure there is a slight decrease in the cleavage product and a decrease in the 115 kDa band for the highest concentrations tested, indicating less active PARP.

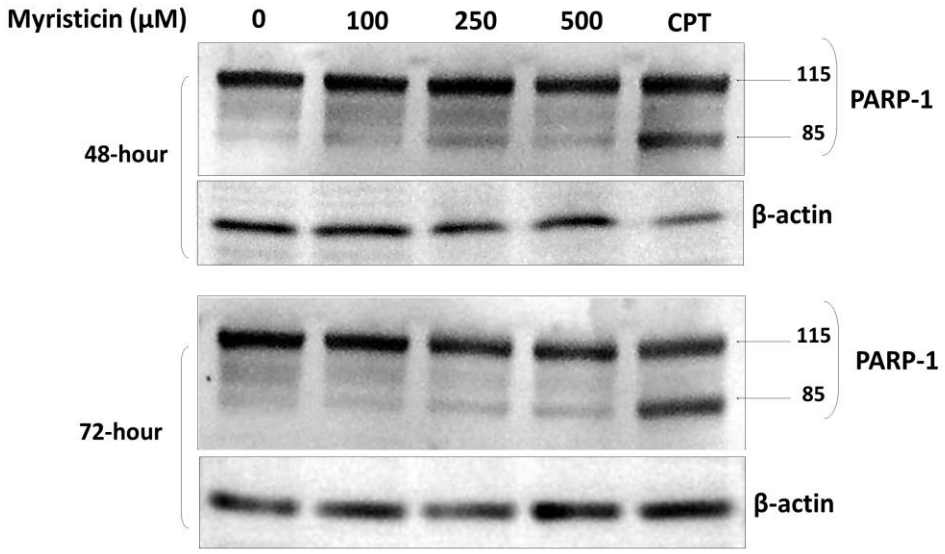


Figure 5.7 PARP cleavage in K562 cells. Cells were harvested after 48 and 72 h incubation with 100, 250 and 500 µM of myristicin. Equal amounts of cell extracts were immune-blotted with the primary antibody for PARP. β-actin was used as an internal control.

5.3.7 *TUNEL assay*

A hallmark of late apoptosis is activation of endonucleases which results in internucleosomal DNA fragmentation that generates a multitude of DNA double-strand breaks with accessible 3'-hydroxyl (3'-OH) groups. This DNA fragments can be detected by the **T**erminal deoxynucleotidyl transferase deoxyuridine triphosphate (d**U**TP) **N**ick **E**nd **L**abelling (TUNEL) assay. So, we performed this assay to understand whether myristicin also induces the latest event of apoptosis. The TUNEL assay identifies apoptotic cells by the terminal deoxynucleotidyl transferase (TdT)-mediated addition of labelled deoxyuridine triphosphate nucleotides (dUTP) to the 3'-OH end of double-stranded DNA breaks. Labelling the dUTP with a probe (DAB) allows for detection by light microscopy. TUNEL is considered a specific assay for positively identifying apoptotic cells based on the finding that necrotic cells have an order of magnitude fewer DNA breaks than apoptotic cells (Kleinberg and Davidson, 2009). TUNEL positive cells (Figure 5.8 A); in brown) were observed after a 48 h period exposure to 50 µM and above of myristicin (Figure 5.8). The percentage of TUNEL labelled cells for myristicin was 15.81% and 18.02% after incubation with 500 and 750 µM, respectively. Almost no DNA fragmentation was observed in control cells. Quantification of cells

positively labelled, showing DNA strand breaks generated during apoptosis, confirmed that myristicin induces apoptosis in K562 cells ($P < 0.05$, ANOVA). At 72 h incubation the number of viable cells was too low to enable the evaluation of TUNEL positive cells (data not shown).

5.3.8 *Effect of myristicin on the expression of DNA damage response genes*

Exposure of K562 cells to 100 μM for a short period of 6 h induced cytochrome c release from the mitochondria. To further assess if alterations in gene expression precedes apoptosis we performed a gene expression analysis for genes involved in the DNA damage response, namely cell cycle, DNA repair, and apoptosis using the RT^2 Profiler™ PCR Gene Array. The Gene Array (using SYBR Green) allows us to assess global patterns of gene expression variation, and to perform an initial screening of genes that could be of interest. In Table 5.2 we indicate the list of genes affected by exposure to myristicin, in **bold** we evidence genes corresponding to ≥ 3-fold differences. Figure 5.9 A) is a volcano plot where the black line indicates fold changes of 1 and the dotted lines indicate fold-changes of 3. The results indicate that 35 of the 84 genes studied are significantly down regulated in response to myristicin and 17 of these have fold differences higher than 3. We confirmed the expression analysis of selected genes using individual Taqman assays, the gold standard for RT-PCR expression, and confirmed that all these genes were significantly down regulated (ATM 1.33-fold, ERCC1 1.42-fold, GADD45A 1.69-fold, GADD45G 2.29-fold, RAD50 1.65-fold, RAD51 1.59 fold) except for MRE11A and XRCC3. These results are shown in Figure 5.9 B). MRE11A was down regulated but without statistical significance (1.31-fold), while XRCC3, unlike our results in the Gene Array, was not altered (1.05-fold increase). The reason for the discrepancy observed with the XRCC3 gene is not obvious, but results obtained with Gene Arrays are more prone to error compared to the individual Taqman assay.

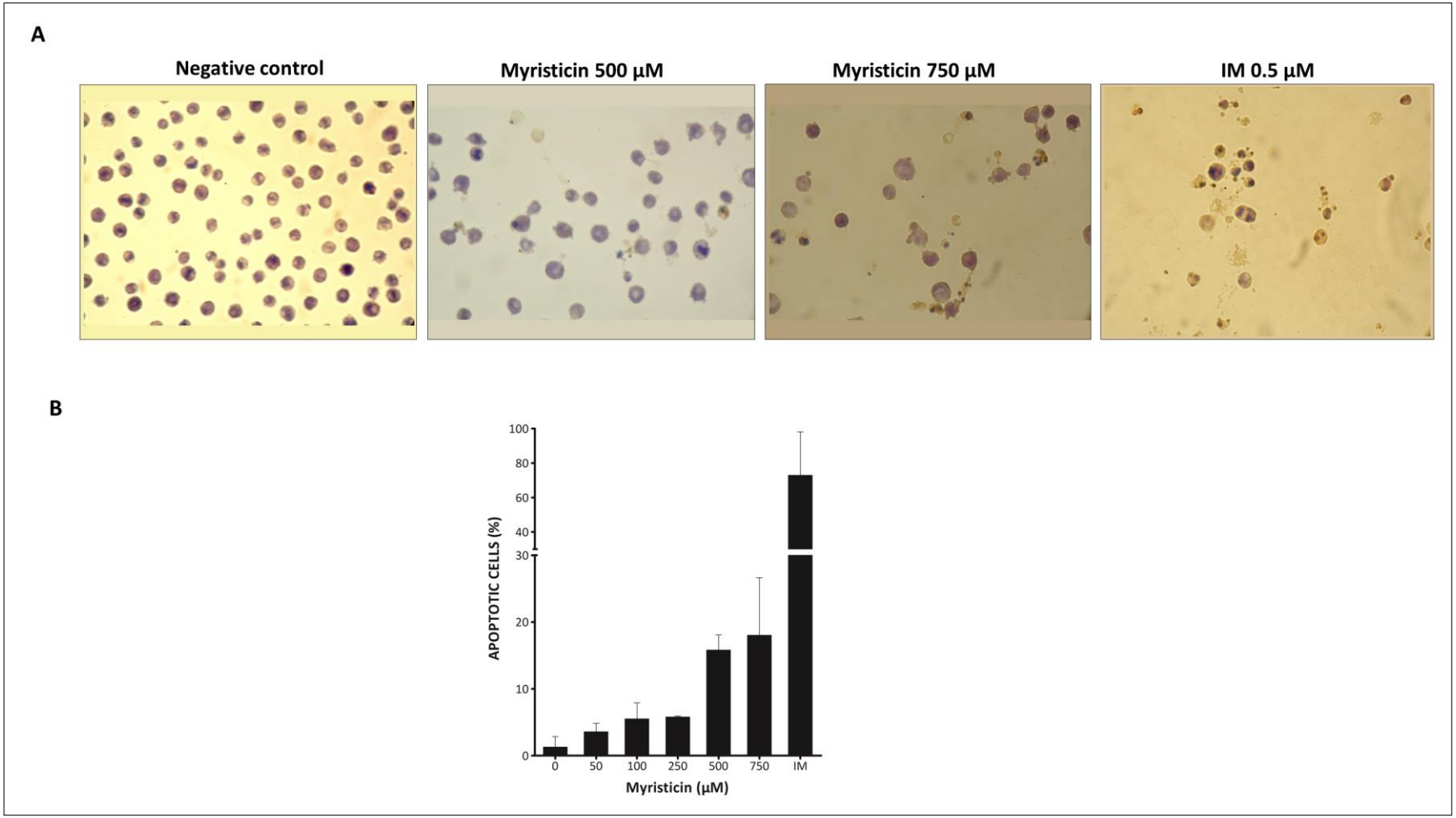


Figure 5.8 Detection of internucleossomal DNA fragmentation by the TdT-mediated dUTP-digoxigenin nick end labelling (TUNEL). The TUNEL assay was performed using the ApopTag Plus Peroxidase In situ apoptosis Detection Kit. (A) Images show analysis of TUNEL positive (brown) or negative (blue) K562 cells after exposure to 500 and 750 µM of myristicin. (B) Data presented for K562 cells after exposure to myristicin for 48 h with a range of concentrations from 50 to 750 µM. Imatinib (IM 0.5 µM) was used as positive control. Induction of apoptosis was significant ($P < 0.05$, ANOVA).

Table 5.2 Gene expression fold-change in DNA damage response pathways induced by 100 µM of myristicin during a 6 h period in Human K562 cells. P values are shown in Italic. *P* values ≤ 0.05 and fold changes higher than 3 are indicated in bold.

Gene	Fold changes[a]	P value[b]	Gene	Fold changes[a]	P value[b]
ABL1	**-3.15**	***0.048***	MSH2	1.79	*0.248*
ANKRD17	-2.48	***0.049***	MSH3	-1.79	***0.032***
APEX1	-1.82	*0.252*	MUTYH	-1.77	***0.006***
ATM	**-7.29**	***0.004***	N4BP2	-2.49	***0.003***
ATR	-1.13	*0.926*	NBN	-2.13	***0.006***
ATRX	-1.86	*0.056*	NTHL1	-1.62	*0.362*
BRCA1	-1.83	***0.047***	**OGG1**	**-3.70**	***0.004***
BTG2	**-5.78**	***0.006***	PCNA	-1.30	*0.247*
CCNH	1.47	*0.413*	AIFM1	-1.21	*0.654*
CDK7	1.68	***0.018***	PMS1	-1.02	*0.927*
CHEK1	1.21	*0.404*	PMS2	-1.86	***0.010***
CHEK2	-1.46	***0.009***	PMS2L3	-1.45	*0.068*
CIB1	-1.18	*0.610*	**PNKP**	**-5.15**	***0.040***
CRY1	-1.01	*0.998*	**PPP1R15A**	**-12.09**	***0.011***
DDB1	-2.48	*0.096*	**PRKDC**	**-3.61**	***0.016***
DDIT3	1.21	*0.294*	RAD1	1.59	*0.360*
DMC1	-1.94	***0.048***	RAD17	1.62	*0.372*
ERCC1	**-15.84**	***0.006***	RAD18	-1.68	*0.050*
ERCC2	**-4.19**	***0.019***	RAD21	-1.32	*0.342*
EXO1	-1.04	*0.731*	**RAD50**	**-10.40**	***0.004***
FANCG	-2.39	*0.054*	RAD51	-1.04	*0.952*
FEN1	**-3.76**	***0.029***	RAD51L1	-2.17	***0.006***
XRCC6	-1.96	*0.235*	**RAD9A**	**-3.01**	*0.290*
GADD45A	-2.83	*0.201*	RBBP8	1.76	*0.379*
GADD45G	**-4.08**	***0.001***	REV1	-2.04	***0.006***
GTF2H1	1.86	*0.363*	RPA1	-1.49	*0.330*
GTF2H2	-1.34	***0.024***	SEMA4A	-2.93	*0.061*
GTSE1	1.32	*0.444*	SESN1	-1.63	***0.005***
HUS1	-2.79	***0.007***	**SMC1A**	**-3.01**	*0.406*
IGHMBP2	**-3.89**	***0.050***	SUMO1	-1.11	*0.394*
XRCC6BP1	-1.40	*0.288*	**TP73**	**-4.43**	***0.025***
LIG1	**-3.71**	*0.059*	**TREX1**	**-3.85**	***0.037***
MAPK12	-2.87	***0.049***	UNG	1.49	*0.423*
MBD4	1.19	*0.472*	XPA	1.60	*0.355*
MLH1	1.44	*0.286*	XPC	-1.05	*0.565*
MLH3	-2.74	***0.012***	XRCC1	-1.98	*0.395*
MNAT1	1.92	*0.146*	XRCC2	-2.65	***0.001***
MPG	-2.17	*0.364*	**XRCC3**	**-7.17**	***0.002***
MRE11A	**-3.38**	***0.004***	ZAK	-1.73	***0.011***

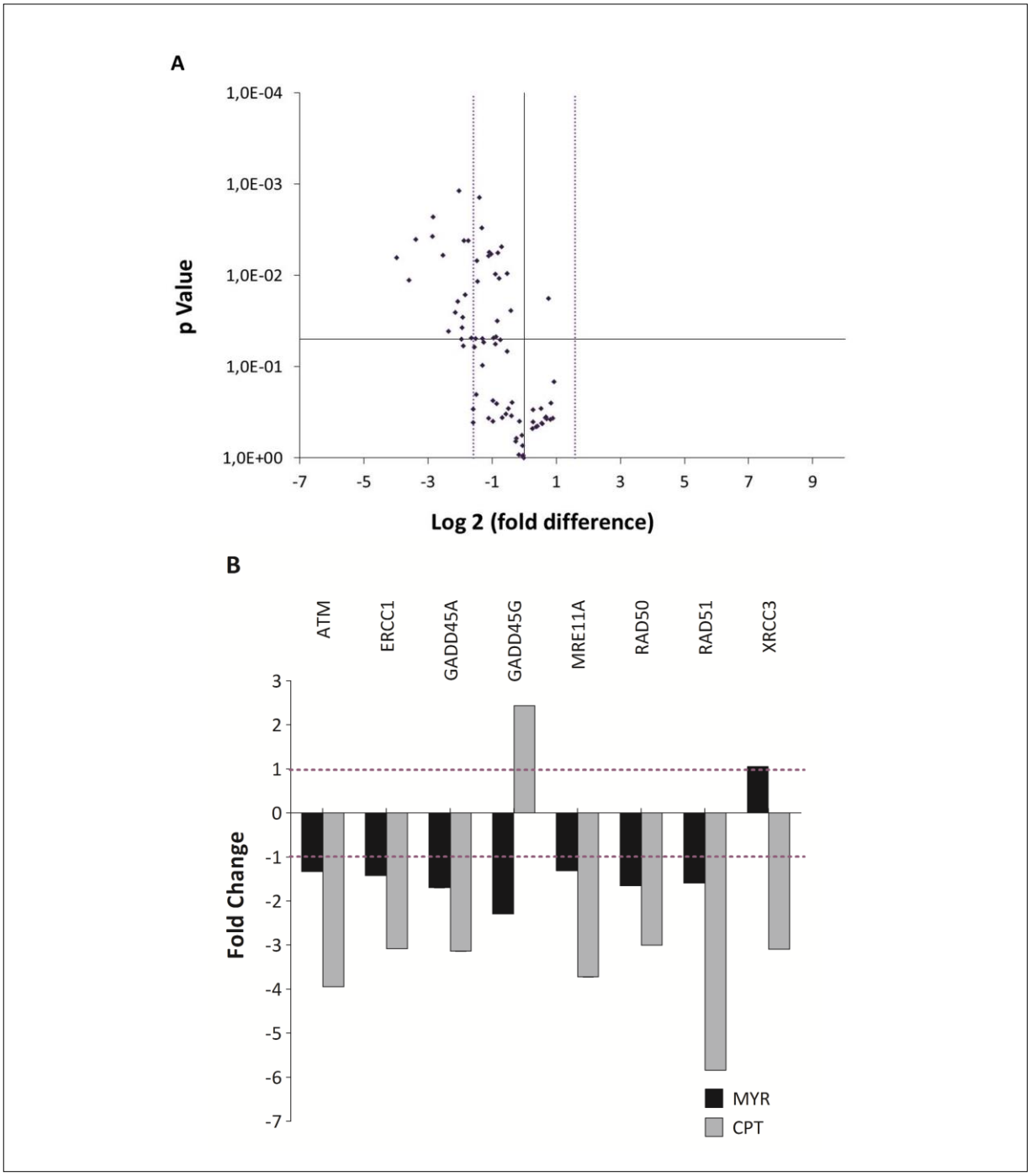


Figure 5.9 RT-PCR results for K562 cells exposed for 6 h to 100 µM of myristicin. (A) Volcano plot of the effects of myristicin treatment on DNA damage response signalling pathway genes assayed by RT^2 Profiler™ PCR Gene Array. (B) Validation of array data by single gene assays. Data indicate fold change in expression as calculated by the $2^{-\Delta\Delta Ct}$ method. Valued shown are mean ± SD of three independent experiments performed in triplicate. The level of significance was determined using the Student *t* test. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

5.4 DISCUSSION

Myristicin is the main active constituent of nutmeg, mace (Jana and Shekhawat, 2010; Muchtaridi et al., 2010) and parsley leaf oil (Wei and Shibamoto, 2007; Zheng et al., 1992), widely used as flavourings. Human consumption of phytochemicals is increasing, due to perceived beneficial health effects and use in CAM, and also widespread increase in consumption of spice flavoured soft-drinks (French et al., 2003; Raffo et al., 2013; Stuckler and Nestle, 2012). Myristicin does not present genotoxicity as demonstrated by our results with the alkaline comet assay and γ-H2AX double strand break detection in Figures 5.2 and 5.3. The UDS assay also gave negative results in hepatocytes derived from male Fischer 344 rats (Hasheminejad and Caldwell, 1994). Additionally, detection of DNA adducts demonstrated that myristicin can bind DNA but to a much lesser extent than other alkenylbenzenes tested, and myristicin-DNA adducts are also less persistent (Phillips et al., 1984; Randerath et al., 1984; Randerath et al., 1993; Zhou et al., 2007). Those results are in agreement with the negative hepatocarcinogenicity results in mice (Miller et al., 1983).

Our previous results indicated that although myristicin was not genotoxic it presented apoptotic activity when compared to other alkenylbenzenes (Martins et al., 2011). Therefore, myristicin was assessed as a possible apoptotic agent in human leukaemia K562 cells. The results obtained show that globally myristicin can induce apoptosis, as characterized by changes in the mitochondrial membrane potential, cytochrome c release, caspase-3 activation, PARP-cleavage and DNA fragmentation.

Dissipation of the mitochondrial transmembrane potential is one of the critical early features of the intrinsic apoptotic pathway (Kleinberg and Davidson, 2009; Ulivieri, 2010) and leads to release of cytochrome-c, as well as the alteration of the balance of proapoptotic and antiapoptotic members of the Bcl-2 family. Cytochrome *c* is considered to be the most prominent proapoptotic signalling protein released from mitochondria (Hengartner, 2000). In our study, release of cytochrome *c* into the cytosol occurred after a 6-h exposure to myristicin, as we can see in Figure 5.5. Release of cytochrome *c* from the mitochondria to the cytoplasm initiates a caspases cascade, whereby cytochrome *c* binds to apoptosis protease-activating factor 1 (Apaf-1) and procaspase-9, generating an intracellular complex known as the apoptosome. Within the

apoptosome, caspase-9 is activated, leading to processing of caspase-3 (Jin and El-Deiry, 2005). The apoptotic machinery has been essentially divided in extrinsic/death receptor and intrinsic/mitochondrial pathways. It is believed that both pathways culminate in activation of a normally latent protease (caspases 8 and 9, respectively), which proceeds to initiate a cascade of proteolysis involving effector caspases responsible for the execution phase of apoptosis (Jin and El-Deiry, 2005). The intrinsic apoptotic programme is more widely implicated as a barrier to cancer pathogenesis (Hanahan and Weinberg, 2011). Since myristicin is lipophilic, it can easily traverse cell membranes and enter the cell. Interaction of myristicin with lipid membranes, including the mitochondrial membrane, is thus more than probable, and can be responsible for its apoptotic activity.

Our data also show that the executor caspases-3 is activated by myristicin in K562 cells (Figure 5.6). Caspase-3 is a predominant effector, and has many cellular targets being PARP one of them (Jin and El-Deiry, 2005; Ulivieri, 2010). PARP cleavage was also seen after 48 h incubation with myristicin (Figure 5.7), with a dose increase of the ~85 kDa cleavage product up to 250 µM and with a decrease for the highest dose tested of 500 µM. The intensity of the cleavage product for the concentration of 250 µM was in agreement with a 2.2-fold increase in caspase-3 activity, confirming that myristicin is not a strong apoptosis inducer when compared with the positive control camptothecin (9.4-fold increase in the caspase activation assay). For the 72 h period there was a slight increase in the cleavage product and a decrease in the 115 kDa band for the higher concentrations tested indicating less active PARP. Thus, PARP was activated and cleaved for the lowest concentrations tested (100 and 250 µM) after a 48 h incubation period, but with the highest concentration and after 72 h myristicin is cytotoxic which prevented us to confirm PARP cleavage.

In addition, we analysed a late feature of apoptosis known to occur during the apoptotic process, internucleossomal cleavage of DNA, measured by the TUNEL assay. TUNEL-positive cells were observed after incubating K562 cells with myristicin for 48 h (Figure 5.8), although the % of apoptotic cells does not exceed 30%, indicating once again that myristicin is not as strong an apoptotic agent as camptothecin. As with the PARP-1 cleavage assay, for the time period of 72 h we could not assess cells possibly due to the toxicity presented by myristicin (data not shown).

Studies regarding the biological effects of myristicin are scarce. In one study, after analysing mace extracts, Ozaki et al. suggested that the active extract myristicin had anti-inflammatory proprieties, inhibiting the edema induced by carrageenan and the increase of the vascular permeability induced by acetic acid (Ozaki et al., 1989). Myristicin was an effective inhibitor of B[a]P-induced tumorigenesis in mice (Zheng et al., 1992). It was also reported that myristicin can prevent liver injury caused by lipopolysaccharide, possessing a possible hepatoprotective activity (Morita et al., 2003). A significant induction of glutathione S-transferases (GST) in mice *in vivo* was observed after treatment with myristicin (Ahmad et al., 1997). Furthermore, Lee *et al.* observed an anti-inflammatory effect of myristicin in RAW 264.7 mouse macrophages, presumably related with its inhibition of nitric oxide, cytokines, chemokines, and growth factors (Lee and Park, 2011).

One hallmark of cancer cells is the use of survival pathways to avoid apoptosis that can occur in irreversibly damaged cells. Some phytochemicals e.g. curcumin and resveratrol, can interfere with the survival of malignant cells by targeting targets relevant to the hallmarks of cancer, namely apoptosis (Lee and Park, 2011; Russo et al., 2010). The concept that apoptosis serves as a natural barrier to cancer development has been proven by functional studies.

Nevertheless, available data indicate that some dietary phytochemicals have the potential to deregulate signalling pathways or reinstate checkpoint pathways in damaged cells (Manson et al., 2007), through alterations in gene expression. In particular the induction of the DNA damage response pathway would suggest a potentially deleterious effect. We thus performed a real-time PCR analysis after exposure to myristicin and observed changes in the expression of genes associated with cell cycle, apoptosis and DNA repair. The dose (100 μM) was chosen because it does not induce growth arrest or cytotoxic effects in K562 cells (MTT assay, Figure 5.1). Globally, the expression profile indicated a down regulation of the genes studied (Table 5.2, Figure 5.9 A). We confirmed this down regulation with individual assays for 8 genes and observed a significant under-expression of genes associated with nucleotide excision repair (ERCC1), double strand break repair (RAD50, RAD51), DNA damage signalling (ATM) and stress response (GADD45A, GADD45G) (Figure 5.9 B). Altogether our results suggest a possible pleiotropic effect resulting from exposure to myristicin. Since up regulation of DNA repair genes occurs in response to DNA

damage, a down regulation of DNA repair genes would be expected if a protective effect is induced. Indeed, recent results with the flavonoid apigenin, which exhibits anti-proliferative and anti-carcinogenic activities, showed a down regulation of DNA repair genes, while increasing the number of apoptotic cells (Arango et al., 2013).

Overall, we demonstrate that myristicin, a natural compound found in nutmeg, mace and parsley leaf oil, in the conditions tested, can alter mitochondrial membrane function, induce apoptosis in human leukaemia K562 cells and down regulates DNA damage response genes. Regular consumption of natural sources such as spices and drinks do not lead to high blood levels of myristicin, thus more studies should be performed with lower concentrations and co-treatments with this flavouring. Also care should be taken when using nutmeg in CAM, where dosage cannot be controlled. This study provides further detail on the molecular mechanism underlying the biological activity of myristicin.

5.5 REFERENCES

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6. Chapter

EPIGENETIC CHANGES AFTER PROLONGED EXPOSURE TO ALKENYLBENZENES: AN IMPORTANT SIGNATURE OF POTENTIAL TOXICOLOGICAL EFFECTS

ABSTRACT

Alkenylbenzenes are a family of natural compounds found in spices, such as nutmeg, clove and anise. Our previous data indicates that some members of the alkenylbenzene family can influence gene expression, i.e. the DNA damage response pathway. On the other hand, it has been described that some dietary phytochemicals can affect epigenetic patterns and deregulates signaling pathways through alterations in gene expression, with a close link between gene expression and DNA methylation. DNA methylation is a hallmark of cancer cells and is characterized by regional CpG island hypermethylation and global genomic hypomethylation. Global hypomethylation induces both genomic activation and instability of proto-oncogenes. Promoter hypermethylation causes tumour suppressor gene silencing, and tends to occur as an early event in carcinogenesis. By definition epigenetic alterations are dynamic, reversible and susceptible to exogenous factors, which are similar to our exposure to dietary compounds. In the present study, as a proof of concept, we analysed methylation alterations in several genes induced by prolonged exposure (15 days) to a low dose (10 μM) of the alkenylbenzenes eugenol and elemicin or by exposure to the isoflavone genistein, in MCF-7 cells. Our preliminary results demonstrated that these compounds may alter methylation patterns. RASSF1 was demethylated by eugenol and the mRNA expression for this gene was enhanced. Eugenol also induced genotoxicity after prolonged exposure, measured by γ-H2AX. This results highlight the importance of prolonged exposure studies in what regards epigenetics events induced by food compounds, as well as their genotoxic effects.

6.1 INTRODUCTION

Epigenetic changes are reversible heritable changes that are not due to alterations in the DNA sequence but can influence gene expression (Holliday, 2006; Meeran et al., 2010; Shankar et al., 2016). Epigenetic modifications include changes in the DNA methylation pattern, posttranslational histone modifications and variations in the expression of non-coding microRNA (miRNA) (Busch et al., 2015), all involved, in some way, with processes regarding cell differentiation, DNA repair and replication, and in stem cell biology (Sincic and Herceg, 2011). DNA methylation is a major epigenetic modification that is strongly involved in the physiological control of genome expression (Alokail and Alenad, 2015). As for the repression of transcription, in maintaining the conformation and integrity of chromosomes as well as in the genome's defense mechanism against potentially damaging mobile genetic elements (Herceg and Hernandez-Vargas, 2011). This epigenetic event consists of a covalent addition of a methyl group (-CH3) on the cytosine pyrimidine ring in DNA by a number of DNA methyltransferases occurring almost exclusively at cytosines that are located 5' to a guanine in a CpG dinucleotide. The DNA methyltransferases involved can essentially be of two types: *de novo* (DNMT3 family) and maintenance (DNMT1) methyltransferases. DNMT1 is the primary maintenance enzyme that preserves existing methylation patterns following DNA replication by adding methyl groups to corresponding daughter strands at the hemi-methylated CpG sites. DNMT3a and DNMT3b preferentially target unmethylated CpGs to initiate *de novo* methylation; they are highly expressed during embryogenesis but less expressed in adult tissues (Alokail and Alenad, 2015; Herceg and Hernandez-Vargas, 2011).

DNA methylation is significantly altered in cancer and is considered a hallmark of cancer; in humans about 70% of CpG sites are methylated, mostly in heterochromatin, and these methylated CpG islands are thought to be important for the control of gene silencing and chromosomal stability. In cancer cells, global hypomethylation is accompanied by the hypermethylation of localized promoter-associated CpG islands, which are usually unmethylated in normal cells, and these alterations, which tend to occur as an early event in carcinogenesis, can lead to chromosomal instability, mutations and reactivation of various oncogenes (Alokail and Alenad, 2015; Vineis et al., 2011). Therefore, it appears that epigenetic deregulation is not

only associated with each step of cancer development and progression, but they occur early in cancer development (Sincic and Herceg, 2011).

In addition to these events non-coding RNAs, namely microRNAs (miRNAs) can be considered part of the epigenetic process because they regulate expression of cellular proteins by affecting mRNA stability and translation. However miRNA can also be epigenetically regulated and in turn can control the expression of various epigenetic modifying enzymes such as DNMTs, histone methyltransferases and histone deacetylases (Meeran et al., 2010; Shankar et al., 2016).

Several authors have described that some dietary phytochemicals can affect epigenetic patterns and deregulate signaling pathways through alterations in gene expression, with a close link between gene expression and DNA methylation (Herceg and Hernandez-Vargas, 2011). More than 10,000 phytochemicals have been described (Russo, 2007), and these include carotenoids, polyphenols, alkaloids, nitrogen-containing and organosulfur compounds. They are widely present in plant-derived foods and beverages (fruits, vegetables and beverages such as tea, wine beer and chocolate), and in many dietary supplements or herbal remedies. Due to the variety of their physiological roles in plant tissues in regulating enzymes involved in cell metabolism and in mechanisms of defence against foreign agents (radiations, viruses, parasites), phytochemicals have been associated to pleiotropic effects in animal cells (D'Incalci et al., 2005; Russo et al., 2010). Several epidemiological studies suggest that a daily intake of phytochemicals can reduce the incidence of several types of cancers (Sporn and Suh, 2002; Surh, 2003), thus suggesting their use in chemoprevention of cancer. Traditionally, these phytochemicals have been utilized in the treatment of various diseases since ancient times. Nevertheless, the mechanisms involved in phytochemical-based chemoprevention are not clearly known. Recently, some of these dietary factors have been shown to regulate the expression of multiple genes through epigenetic modulatory mechanisms, including tumour suppressor genes, oncogenes and miRNAs (Shukla et al., 2014).

As stated before, alkenylbenzenes comprise a family of phytochemicals found mainly in spices, such as nutmeg, clove and anise. Eugenol is frequently used in dentistry practise as a cement material or as a sedative agent and elemicin is one of the main components of nutmeg. Clove and nutmeg are currently used as flavours and in CAM. Our previous data (chapter V) indicates

that some members of the alkenylbenzene family can influence gene expression, i.e. DNA damage response pathway. As mentioned, one mechanism by which these natural compounds may alter gene expression is through epigenetic mechanisms. Thus, the focus of this chapter is to discuss our understanding of epigenetic events (DNA methylation changes) mediated by the alkenylbenzene family, as a proof of concept approach, since no studies of this nature were identified in the literature. For that matter we also analyzed the isoflavone genistein (soy-bean) described as having epigenetic action and DAC, a known demethylating agent.

Most studies in toxicology have analysed the exposure of high doses of xenobiotics for a short period of time, typically during 1-2 cell cycles. Although followed for practical reasons, this methodology does not reveal the biological effects, namely epigenetic effects, after a prolonged exposure, more in line with chronic exposure to dietary components. Keeping this in mind, we analysed methylation alterations in several genes after prolonged expose (15 days) to a low dose of 10 μM of elemicin or eugenol using human MCF-7 cells, representative of breast adenocarcinoma and MDA-MB 231 metastatic (advanced) breast cancer cells. We also analysed if the prolonged exposure throughout the 15 days could induce cytotoxicity and/or genotoxicity using the Trypan Blue viability assay and measuring H2AX phosphorylation by western blot.

6.2 MATERIAL AND METHODS

6.2.1 *Chemicals and Reagents*

Eugenol (CAS Nº 97-53-0, 99.6%, PESTANAL from Sigma-Aldrich), elemicin (CAS Nº 487-11-6, from Toronto Research Chemicals), genistein from *Glycine max*, soybean (CAS Nº 446-72-0, ~98%, from Sigma-Aldrich) and 5-Aza-2'-deoxycytidine (DAC) were purchase from Sigma-Aldrich (Sigma Chemical Co.). DMSO, sodium chloride, potassium chloride and magnesium chloride were obtained from Merck KGaA (Darmstadt, Germany). Nonidet P-40 from USB Corporation (Cleveland, OH, USA) and the protease inhibitor cocktail from Roche Diagnostics (Mannheim, Germany). Glycine, SDS, bovine serum albumin (BSA) and Bradford were obtained

from BioRad and Trypan Blue 0.4% from Gibco (ThermoFisher scientific). All the other reagents, unless otherwise specified, were obtained from Sigma-Aldrich (St. Louis, MO, USA).

6.2.2 *Cell Culture*

Human breast cancer cells MCF-7 and MDA-MB 231 (DSMZ, Germany) were routinely maintained in 175 cm^2 culture flasks in Dulbecco's modified Eagle's medium (DMEM; sigma) supplemented with 10 % fetal bovine serum and 1 % antibiotic solution (penicillin–streptomycin) at 37 ºC, under an atmosphere containing 5% CO_2. MCF-7 cells medium were supplemented also with 0.01 mg/ml of insulin solution from bovine pancreas.

MCF-7 and MDA-MB 231 cells were exposed to 10 µM of each alkenylbenzene or genistein during a period of 15 days, with renovation of the culture medium (with chemical) and trypsinization every three days. In each renovation day (3^{th}, 6^{th}, 9^{th}, 12^{nd} and 15^{th} day) samples for extraction of nucleic acids and protein extraction (with immediate extraction) were withdrawn. Parallel cultures were also maintained with DMSO at 0.1% v/v and the demethylation agent DAC was also tested during a period of 5 days at a concentration of 2.5 µM. Higher incubation periods were considered toxic for these cells and were not pursued further.

6.2.3 *Trypan Blue Viability Assay*

The number of viable cells, present in the cell suspension obtained after each 3 days of exposure, was measured using the trypan blue exclusion assay. This assay is based on the principle that live cells possess intact cell membrane that exclude certain dyes such as trypan blue, so viable cells have a clear cytoplasm whereas non-viable cells appear with a blue cytoplasm. Briefly, cells were centrifuged, and the cell pellet was resuspended in 1 ml of medium. Twenty microliters of cell suspension were mixed with 20 µL of 0.4 % trypan blue. The trypan blue/cell mixture was applied in to a hemacytometer and cells were observed under a light microscope (Nikon, Dialux 20). The viable and non-viable cells were counted and the percentage of viable cells was calculated as following:

$$Viability\ (\%) = \frac{viable\ cells\ per\ ml}{total\ number\ of\ cells\ per\ ml} \times 100$$

6.2.4 *Western blot analysis*

6.2.4.1 *Preparation of cytosolic and nuclear lysates:* The method was performed essentially as described by *Bhushan et al*. (Bhushan et al., 2007) with minor modifications. Briefly, cells were washed in ice-cold PBS and resuspended in ice-cold cytosol extraction buffer (10 mM HEPES/KOH at pH 7.9, 2 mM $MgCl_2$, 0.1 mM EDTA, 10 mM KCl, Nonidet 1%, 1 mM dithiotheritol (DTT), protease inhibitor cocktail (1% v/v) and phosphatase inhibitor cocktail (1% v/v)) at a final concentration of 1×10^7cells/400 µl of buffer. After 5 min incubation on ice, the preparations were vortex for 5 seconds, and then centrifuged at 4000 ×g for 5 minutes, the supernatants were kept as cytosolic fractions. The pellets obtained were resuspended in approximately 100 µl of ice-cold nucleus lysis buffer (50 mM HEPES/KOH at pH 7.9, 300 mM NaCl, 50 mM KCl, 0.1 mM EDTA, glycerol (10 % v/v), 0.5 mM PMSF, 1mM DTT, protease inhibitor cocktail (1% v/v) and phosphatase inhibitor cocktail (1% v/v)) and preparations were vortex for 15 seconds and incubated 20 min on ice, with continuing vortex, followed by a 5-min centrifugation at 15,000 ×g at 4 ºC. The supernatants were collected as nuclear fractions.

6.2.4.2 *Protein quantification:* Protein was measured using the Bradford Method (Bio-Rad Protein Assay; Bio-Rad laboratories, München, Germany), according to the manufacturer's microassay procedure.

6.2.4.3 *Immunoblotting technique:* Western blot was performed using Mini-Protean®TGX™ Precast gels (Bio-Rad, Hercules, CA, USA), according to the manufacturer's procedure. Each well was loaded with 40 - 60 µg of cytosolic or nuclear proteins. Proteins were then transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore, USA). Protein detection was performed using primary antibodies: anti-β-actin (1:1000) from Santa Cruz Biotechnology (Santa Cruz, CA, USA), anti-TATA binding protein TBP (1:1000) from Abcam (Abcam, Cambridge, UK), anti-phospho-ATM (Ser1981) (1:1000) and anti- DNMT1 (D59A4) (1:1000) from Cell Signaling, Anti-phospho-Histone H2AX (Ser139) and anti-ATM (clone Y170) from Millipore. PVDF membranes were treated with the WesternDot™ 625 Western blot kit (Invitrogen Molecular Probes, Eugene, USA), according to the manufacturer's procedure. Finally, images were captured using ChemiDoc™ Touch Imaging System from BioRad. Each Western blot was performed in at least two independent assays.

6.2.5 *Methylation Specific PCR (MSP)*

DNA obtained from each sample (DNA/RNA MiniKit, Qiagen) was subjected to sodium bisulfite modification using the EZ DNA Methylation-Gold ™ Kit (ZYMO Research, CA, USA). Methylation specific PCR was performed using the Zymo*Taq* DNA Polymerase (ZYMO Research, CA, USA) according to the manufacturer's instructions.

Bisulfite conversion was done by adding 130 µl of CT conversion reagent to 20 µl of DNA sample (300 ng), all steps forward were done according to the manufacturer's instructions. The obtained bisulfite converted DNA was stored at -20 ºC until use, or used immediately. For the PCR reaction, two separated mixes were prepared using primers for unmethylated and methylated sequences (described in tables 6.1 and 6.2). The mix only differed in the primer sequences used. Thus, 50 µl reaction mix were prepared, and 50 ng (1 µl) were used for the reaction, all was done according to the manufactures instructions. The PCR conditions were: initial denaturation at 95 ºC for 10 minutes, 40 cycles of denaturation at 94 ºC for 30 seconds, annealing temperature was according to each primer (tables 6.1 and 6.2) for 30 seconds, and extension at 72 ºC for 30 seconds, followed by a final extension at 72 ºC for 7 minutes and cooling to 4ºC. The MSP products were resolved on 2 % agarose gels and visualized by GreenSafe premium (nzytech genes). Images were captured using ChemiDoc™ Touch Imaging System from BioRad. Human methylated and non-methylated DNA (Biognostica) were used as controls for the reaction, and at least two independent experiments were performed.

6.2.6 *Real-time PCR*

Total RNA (1 µg) was extracted from cells (Kit DNA/RNA, Qiagen) obtained in the different time periods (3, 6, 9 12 and 15 days exposure). After assessing RNA yield and quality, total RNA was converted to cDNA using the High Capacity RNA-to-cDNA kit (Applied Biosystems) according to the manufacturer's instructions. Expression was determined using Taqman assays (Taqman®Universal Master Mix II) from Applied Biosystems (Gene RASSF1, ID: Hs00200394_m1). Relative gene expression level was normalized with GADPH (4352934E; Applied Biosystems). Differences between cells exposed to eugenol, elemicin or genistein and non-exposed (DMSO 0.1% v/v) cells are presented as fold change based on calculation of $2^{-\Delta\Delta Ct}$. RT-PCR was performed in triplicate and repeated in three independent experiments. PCR was

performed on the 7300 RT PCR systems (Applied Biosystems). All assays were performed according to protocols from the manufacturer.

Table 6.1 Oligonucleotide data for the genes and miRNAs used in the prolonged exposure to elemicin and eugenol.

Genes/ miRNAs	USP Sequence	MSP Sequence	Annealing (ºC)
ABCB1-50	F 5’ TATTGTGGAGTGTGGGTT 3’ R 5’ CCAATCAACAAACTCCCAACAA 3’	F 5’ TATTGCGGAGTGCGGGTC 3’ R 5’ TCGACGAACTCCCGACGA 3’	57
miR 124-1-170	F 5’ TTTGGTTGGGTTGGTAGAATT 3’ R 5’ CACAACAACCACACATATTCTAAA 3’	F 5’ GGTTGGGTCGGTTGAATC 3’ R 5’ AAGACCACGCGTATTCTAAA 3’	62
GSTP1	F 5’ GATGTTTGGGGTGTAGTGGTTGTT 3’ R 5’ CCACCCCAATACTAAATCACAACA 3’	F 5’ TTCGGGGTCTAGCGCTCGTC 3’ R 5’ GCCCCAATACTAAATCACGACG 3’	60
RASSF1	F 5- GGT TGT ATT TGG TTG GAG TG -3 R 5- CTA CAA ACC TTT ACA CAC AAC A -3	F 5- GTT GGT ATT CGT TGG GCG C-3 R 5- GCA CCA CGT ATA CGT AAC G-3	56
miR LET7A3	F 5' GAGGAGATGGTATGTTTGTGAAGTTG 3' R 5' AAACATACAAATACCCACCCTACTCA 3'	F 5' GACGGTACGTTCGTGAAGTCG 3' R 5' CATACGAATACCCACCCTACTCG 3'	55

6.2.7 *Statistical Analysis*

To analyze differences in the relative expression of RASSF1A gene between the controls and treated samples we used the two-way ANOVA with Borferroni post-test (*P* value < 0.05). All statistical analyses were performed with the GraphPad Prism 5 software (GraphPad Software, Inc.).

Table 6.2 Oligonucleotide data for the genes and miRNAs used in the MCF-7 and MDA-MB 231 cell lines scan.

Genes/ miRNAs	USP Sequence	MSP Sequence	Annealing (ºC)
MLH1	F 5’ TTTTGATGTAGATGTTTTATTAGGGTTGT 3’ R 5’ ACCACCTCATCATAACTACCCACA 3’	F 5’ ACGTAGACGTTTTATTAGGGTCGC 3’ R 5’ CCTCATCGTAACTACCCGCG 3’	55
DAPK1	F 5’ GGAGGATAGTTGGATTGAGTTAATGTT 3’ R 5’ CAAATCCCTCCCAAACACCAA 3’	F 5’ GGATAGTCGGATCGAGTTAACGTC 3’ R 5’ CCCTCCCAAACGCCGA 3’	57
MYB	F 5’ GGTTTGTTTAGGAAAAGGTGTT 3’ R 5’ CTACCAAACDAACCAAATCACC 3’	F 5’ TTTGTTTAGGAAAAGGCGTC 3’ R 5’ CTACCGAACTAACCGAATCG 3’	60
miR 126-29	F 5’ ATTTTGGAAGATGTTATGTTTTT 3’ R 5’ TACCATAAACAACACATTATTAC 3’	F 5’ TTGGAAGACGTTACGTTTTC 3’ R 5’ TACCGTAAACGACGCATTAT 3’	47 51
MGMT	F 5’ CCAAATATACTAAAACAACCCACA 3’ R 5’ TGAATTTTTTTGGGAGTGAGGT 3’	F 5’ CGAATATACTAAAACAACCCGCG 3’ R 5’ GTATTTTTTCGGGAGCGAGGC 3’	55
BRCA1	F 5’ TTGGTTTTTGTGGTAATGGAAAAGTGT 3’ R 5’ CAAAAAATCTCAACAAACTCACACCA 3’	F 5’ TCGTGGTAACGGAAAAGCGC 3’ R 5’ AAATCTCAACGAACTCACGCCG 3’	57
miR 124-2	F 5' GGTGTATTTTGGGGTTTTTGT 3' R 5' TACAAACAAAACCCTCTACACA 3'	F 5' CGTATTTTGGGGTTTTTGC 3' R 5' TACGAACGAAACCCTCTACG 3'	58
miR 124-3	F 5' GTTGGGATTGGTAATTATGTTT 3' R 5' CAAAAAAACACTCAAACTATTC 3'	F 5' CGGGATTGGTAATTACGTTC 3' R 5' CGAAAAAACGCTCGAACTAT 3'	56
miR 219-2	F 5' TTTGTTTTTTTGTGGTTGAGTT 3' R 5' CACAAACACTACAAATAACCCA 3'	F 5' CGTTTTTTTGTGGTTGAGTC 3' R 5' CACGAACGCTACAAATAACC 3'	50
miR 24-1	F 5' TTTATGGAGTTTTTAGTTGAGGT 3' R 5' CCTCAAACACTTACAAACACAAA 3'	F 5' ACGGAGTTTTTAGTTGAGGC 3' R 5' TCGAACACTTACAAACACGA 3'	52

6.3 RESULTS

6.3.1 *Viability*

Cell viability was checked by trypan blue dye exclusion assay. Treatment of MCF-7 cells or MDA-MB 231 cells with all the compounds for 15 days displayed no significant alteration in cell viability. More than 80% of the cells were viable after treatment with eugenol, elemicin or genistein, as we can see in Figures 6.1 and 6.2.

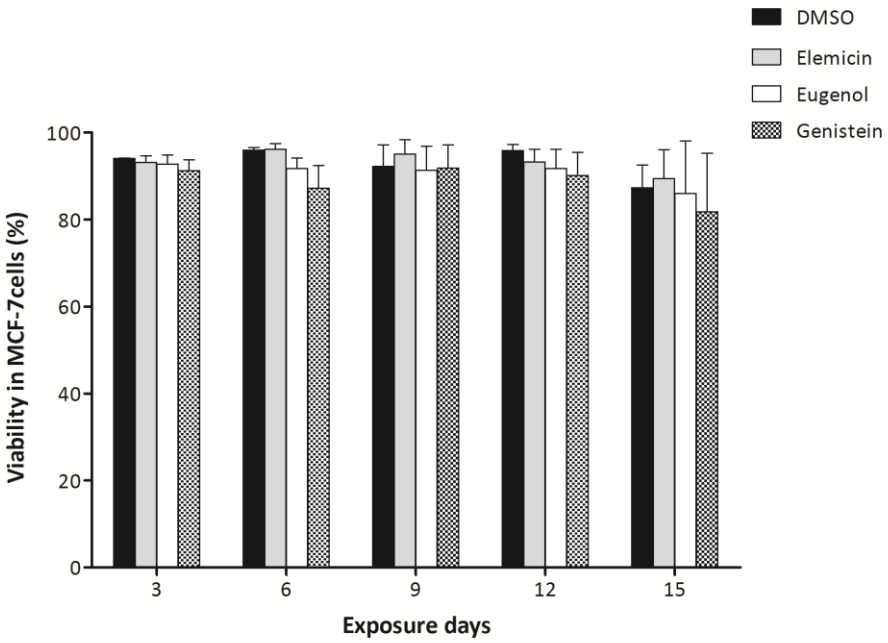

Figure 6.1 Viability assay using Trypan Blue in MCF-7 cells exposed to the alkenylbenzenes elemicin and eugenol and to the isoflavone genistein.

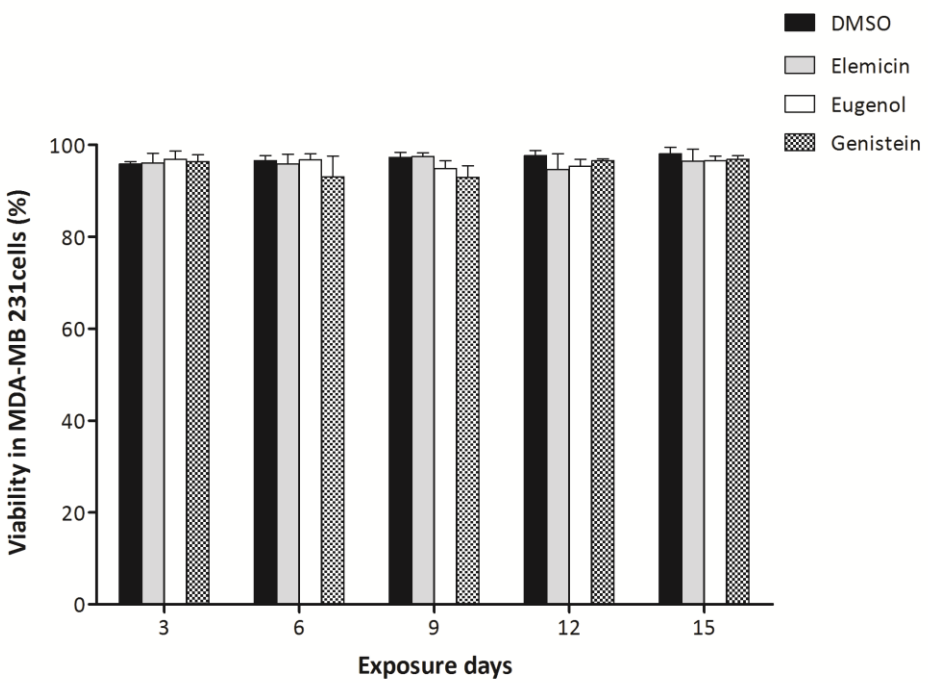

Figure 6.2 Viability assay using Trypan Blue in MDA-MB 231 cells exposed to the alkenylbenzenes elemicin and eugenol and to the isoflavone genistein.

6.3.2 Genotoxic response after prolonged exposure to alkenylbenzenes and genistein: γ-H2AX and p-ATM detection by western blot

As we can see in Figures 6.3 and 6.4 both eugenol and elemicin induced phosphorylation of the protein H2AX in a time dependent manner. Furthermore, ATM was also phosphorylated at longer exposure periods. As both H2AX and ATM are key genes in the DNA damage response pathway, these results indicate that prolonged exposure to these compounds may elicit a genotoxic response.

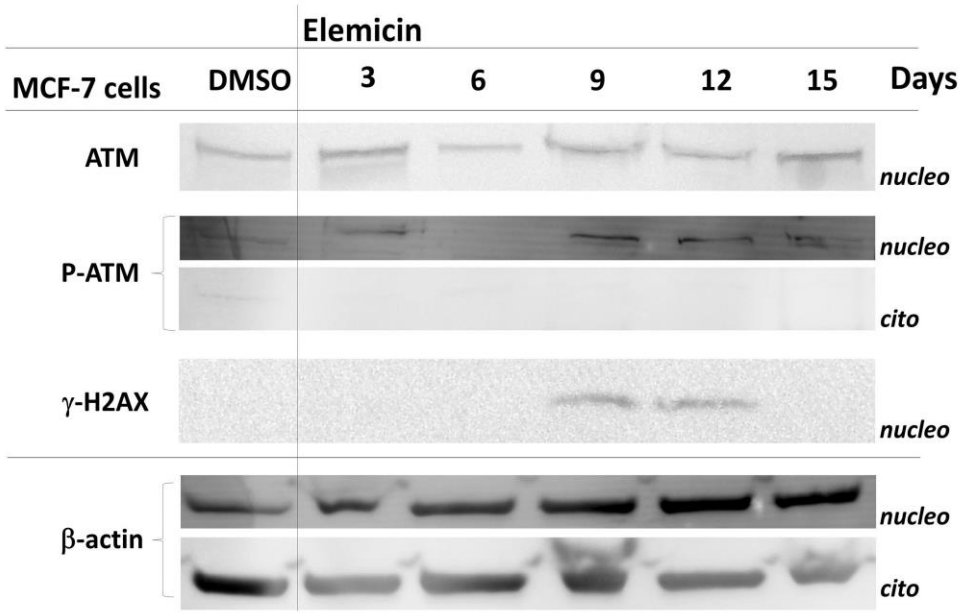

Figure 6.3 Western Blot for detection of γ-H2AX, ATM and P-ATM, in MCF-7 cells exposed to elemicin. β-actin was used as internal control. (nucleo=nucleus; cito=cytoplasm)

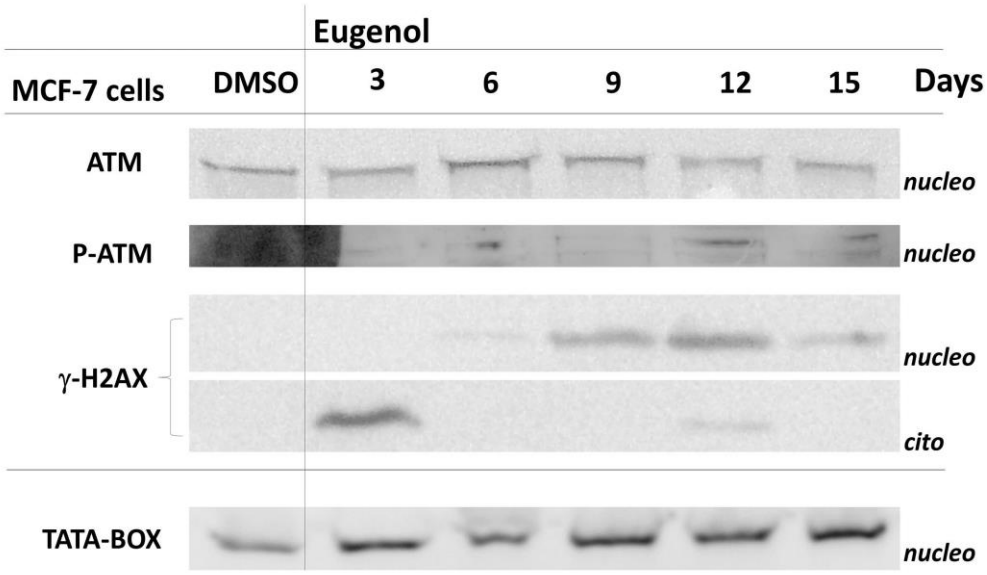

Figure 6.4 Western Blot for detection of γ-H2AX, ATM and P-ATM, in MCF-7 cells exposed to eugenol. TATA-BOX was used for the nuclear extracts internal control. (nucleo=nucleus; cito=cytoplasm)

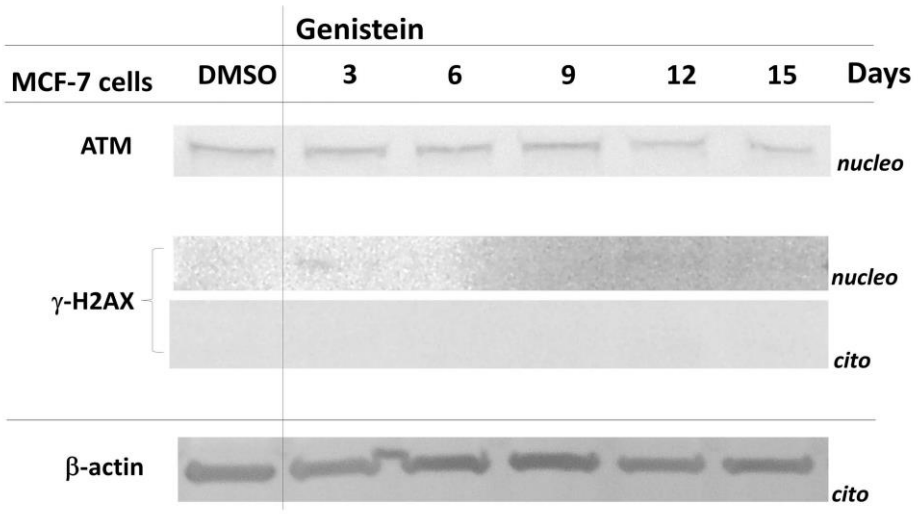

Figure 6.5 Western Blot for detection of γ-H2AX, ATM and P-ATM, in MCF-7 cells exposed to genistein. β-actin was used as internal control. (nucleo=nucleus; cito=cytoplasm)

6.3.3 Methylation status of several genes and miRNAs in MCF-7 and MDA-MB 231 cells

A number of genes and miRNAs were studied for their basal methylation status in mammalian MCF-7 and MDA-MB 231 breast cancer cells, i.e. without exposure to alkenylbenzenes. A summary of results is given in table 6.3. As we can observe in MCF-7 cells, the genes studied present a variable pattern of methylation. Interestingly almost all the genes analyzed are unmethylated in MDA-MB 231 cells, which are metastatic, therefore more transformed than MCF-7 cells. Regarding the miRNAs studied, once again we can observe a variable pattern of methylation, and MDA-MB 231 cells do not present preferential unmethylation compared to MCF-7 cells. Based on the date obtained, the genes RASSF1A, GSTP1 and the miRNAs LET7A3 and 124-1-170 were chosen to ascertain any change in methylation patterns in MCF-7 cells. The choice was based on the fact that these genes in the CpGs Islands studied were highly methylated and we expected that exposure to the alkenylbenzenes could induce demethylation. MCF-7 cells were chosen because they presented a higher number of methylated genes compared to MDA-MB-231 cells.

Table 6.3 Overview of the methylation status of the human breast cancer cells MCF-7 and MDA-MB 231

Genes	MCF-7		MDA-MB 231		miRs	MCF-7		MDA-MB 231	
	M	UM	M	UM		M	UM	M	UM
ABCB1-50					LET7A3				
BRCA-1					24-1				
DAPK1					126-29				
GSTP-1					124-1-170				
MGMT					124-2				
MLH1					124-3				
MYB					219-1				
RASSF1					219-2				

ND – No Data, M – Methylated, UM – Unmethylated. Grey intensity represents methylation patterns (White indicates the CpG has 0% methylation or unmethylation).

6.3.4 Methylation status of the promoters of the genes RASSF1A, GSTP1 and ABCB1 and the miRNAs LET7A3 and 124-1-170

CpG island hypermethylation in the promoter regions plays a critical role in silencing the expression of various genes and miRNAs involved in the carcinogenesis process, such as RASSF1A, GSTP1, miR124-1-170 and miRLET7A3. To determine whether eugenol and elemicin has a demethylating effect on these genes we examined the changes in DNA methylation status in MCF-7 cells following exposure through 15 days to 10 µM of each compound.

As shown in Figures 6.6 and 6.7, treatment with 10 µM of eugenol resulted in demethylation of RASSF1A at 9 and 12 days, while the miR124-1-170 promoter also underwent demethylation after 6 days exposure. Regarding LET7A3 at 3 days of exposure we can observe an increase in demethylation induced by all compounds, but only eugenol maintains unmethylation up to 15 days exposure (Figure 6.8).

GSTP1 is essentially methylated in MCF-7 cells but the treatment with either compound did not elicit marked changes in the methylation status (Figure 6.9). Likewise, the various treatments did not have an effect on the methylation of the promoter of ABCB1, although genistein seemed to increase the levels of unmethylated promoter over time (Figure 6.10).

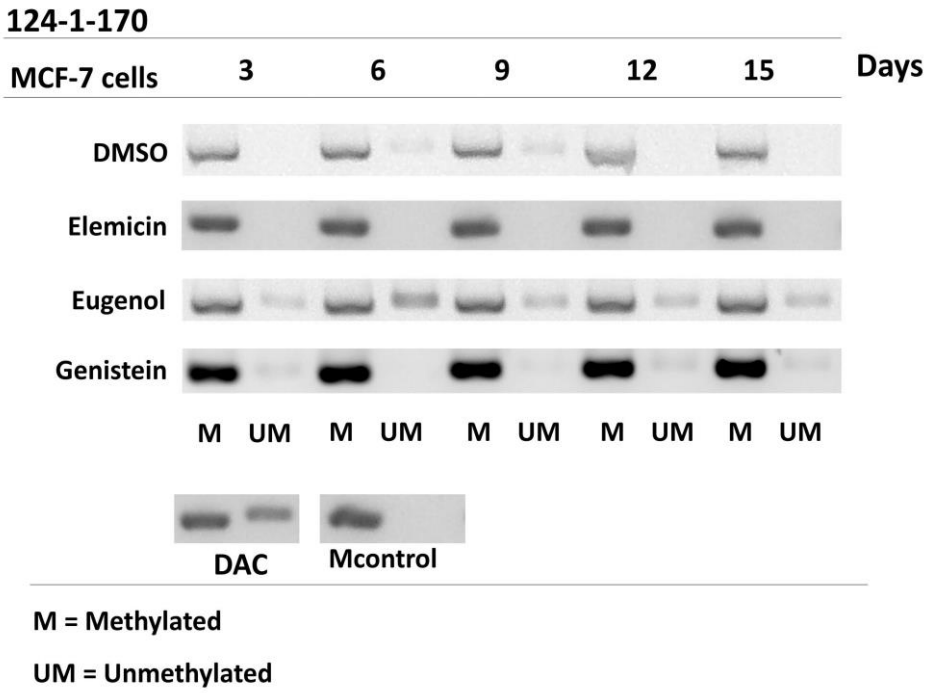

Figure 6.6 MSP for miR124-1-170 in MCF-7 cells exposed to the alkenylbenzenes eugenol and elemicin and to the isoflavone genistein. DAC had a strong demethylation effect. Mcontrol - human methylated control.

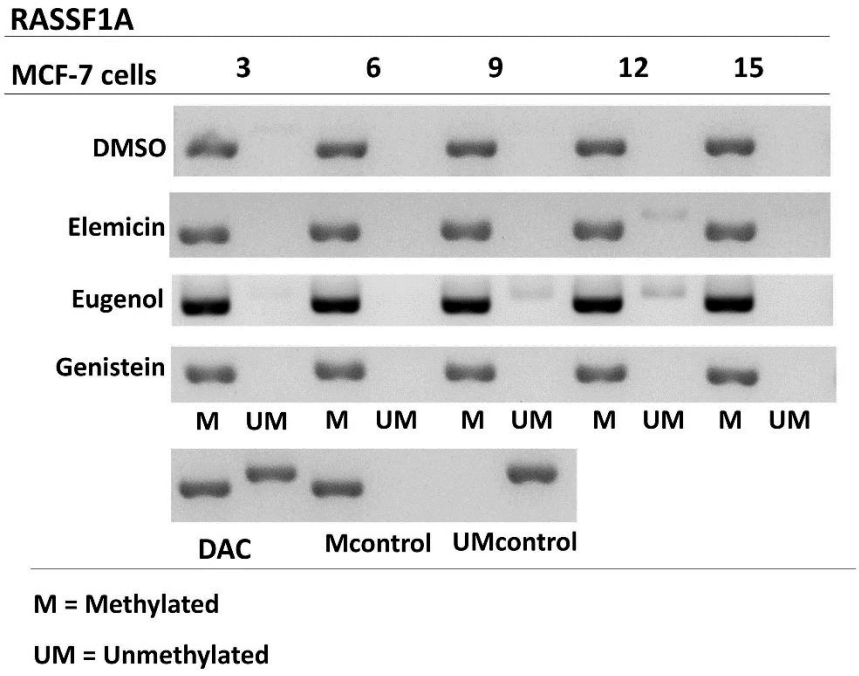

Figure 6.7 MSP for the gene RASSF1A in MCF-7 cells exposed to the alkenylbenzenes eugenol and elemicin and to the isoflavone genistein. DAC had a strong demethylation effect. Mcontrol - human methylated control, UMcontrol – human unmethylated control.

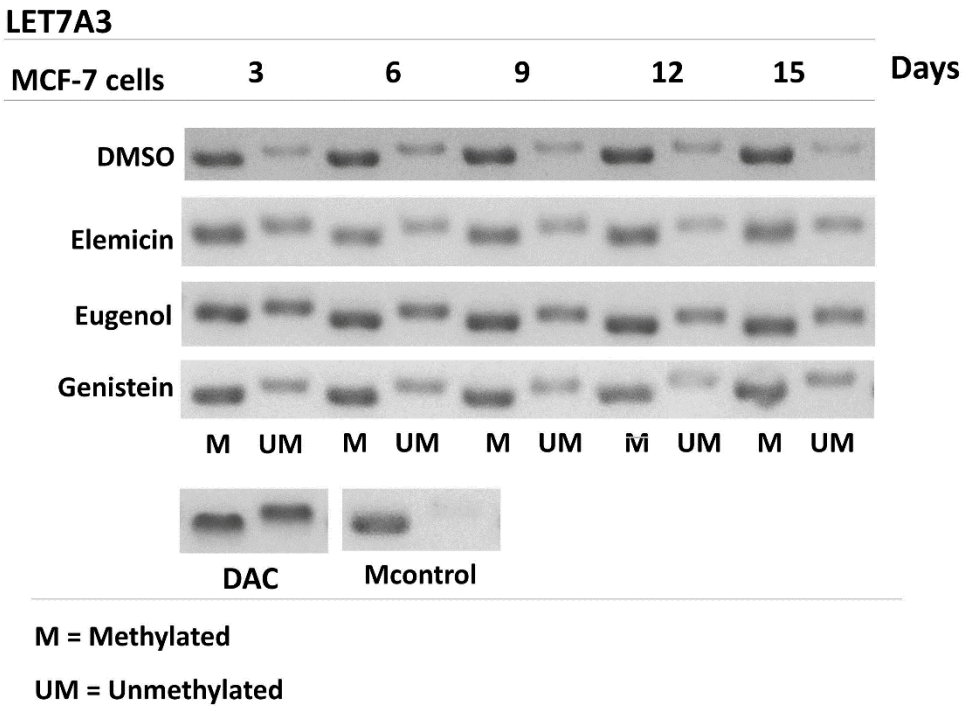

Figure 6.8 MSP for miRLET7A3 in MCF-7 cells exposed to the alkenylbenzenes eugenol and elemicin and to the isoflavone genistein. DAC had a strong demethylation effect. Mcontrol - human methylated control.

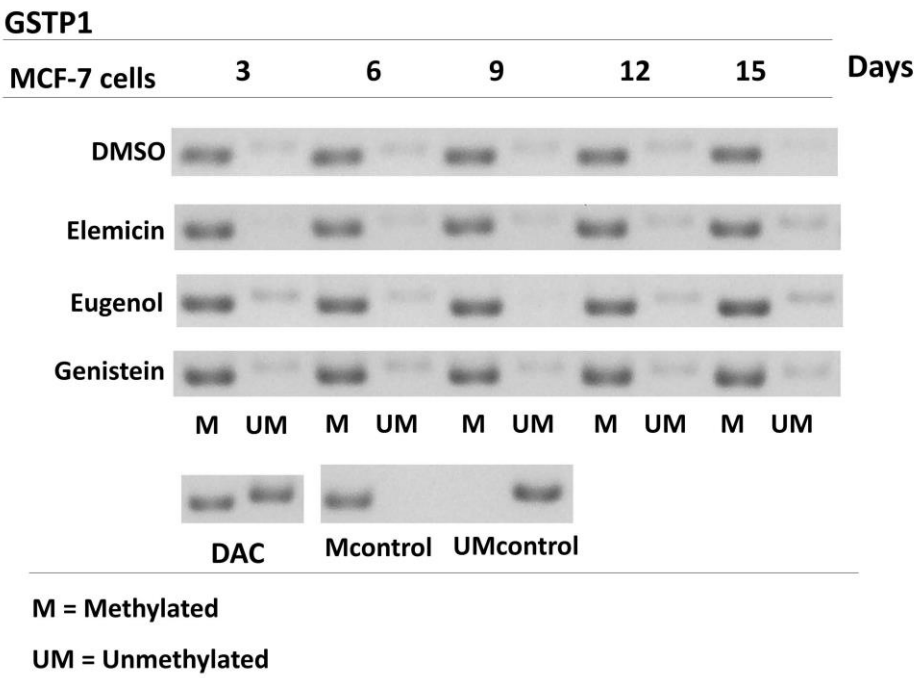

Figure 6.9 MSP for the gene GSTP1 in MCF-7 cells exposed to the alkenylbenzenes eugenol and elemicin and to the isoflavone genistein. DAC had a strong demethylation effect. Mcontrol - human methylated control, UMcontrol – human unmethylated control.

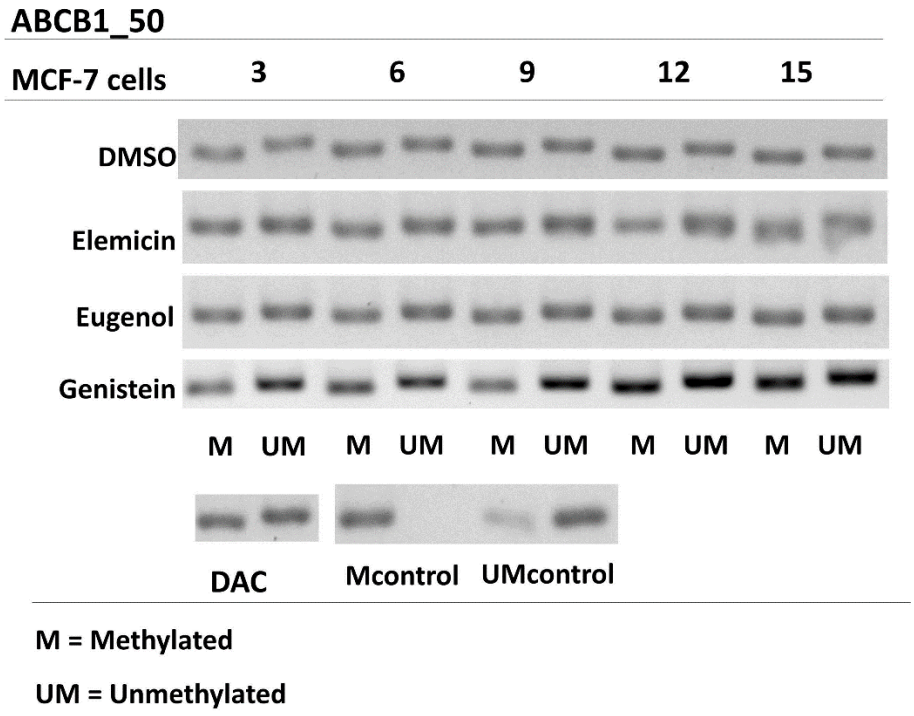

Figure 6.10 MSP for the gene ABCB1-50 in MCF-7 cells exposed to the alkenylbenzenes eugenol and elemicin and to the isoflavone genistein. DAC had a strong demethylation effect. Mcontrol - human methylated control, UMcontrol – human unmethylated control.

6.3.5 DNMT1 Protein expression

Some reports indicate that the expression levels of DNMT1 could be altered after exposure to phytochemicals, thus explaining the different methylation patterns observed in the cell lines studied. Indeed genistein decreases expression of DNMT1 in MCF-7 cells after an exposure to 60 or 100 µM up to 72h (Xie et al., 2014). Thus nuclear extracts were analyzed for differences in DNMT1 expression. The various treatments did not seem to influence DNMT1 protein expression (Figure 6.11). In contrast with published data (Xie et al., 2014) we could not observe any influence of 10 µM of these compounds, although elemicin seems to increase expression at 6 days, but data were not consistent.

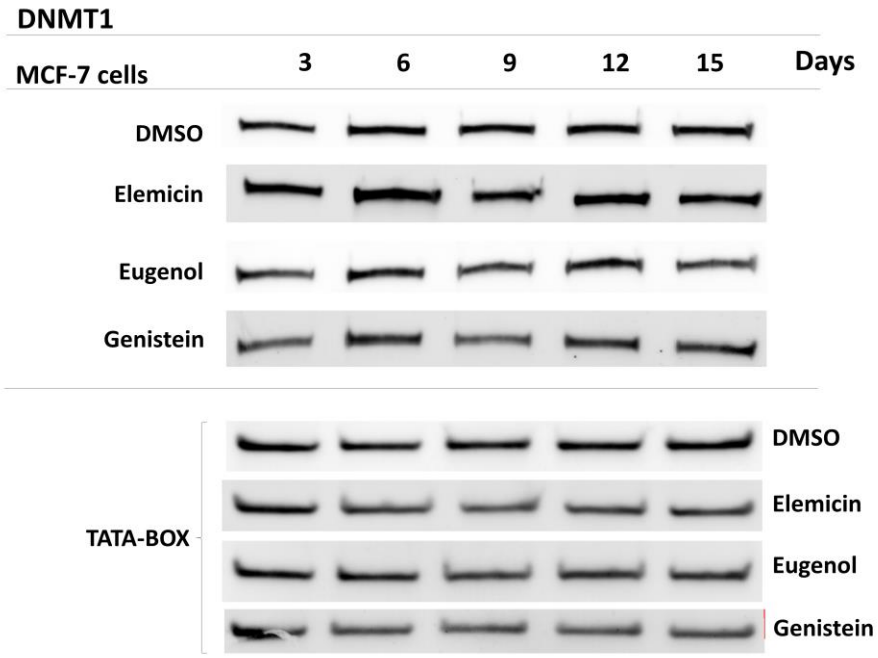

Figure 6.11 DNMT1 protein expression in MCF-7 cells. TATA-BOX was used as internal control for the nuclear extracts.

6.3.6 RASSF1A gene expression after treatment with alkenylbenzenes or genistein

In order to follow-up on the methylation data of RASSF1A presented previously, we performed RT-PCR to assess expression of this gene. Data are presented in Figure 6.12. Although we observe a significant increase of RASSF1A expression after 3 days with eugenol ($P < 0.01$) and genistein ($P < 0.001$), and after 9 days with genistein ($P < 0.01$), these data were not directly related with MSP results (Figure 6.7). This indicates that exposure to alkenylbenzes may alter gene expression, as with the DNA damage response genes presented previously (Chapter V), but we could not directly relate expression with the bands seem with the MSP, especially for genistein.

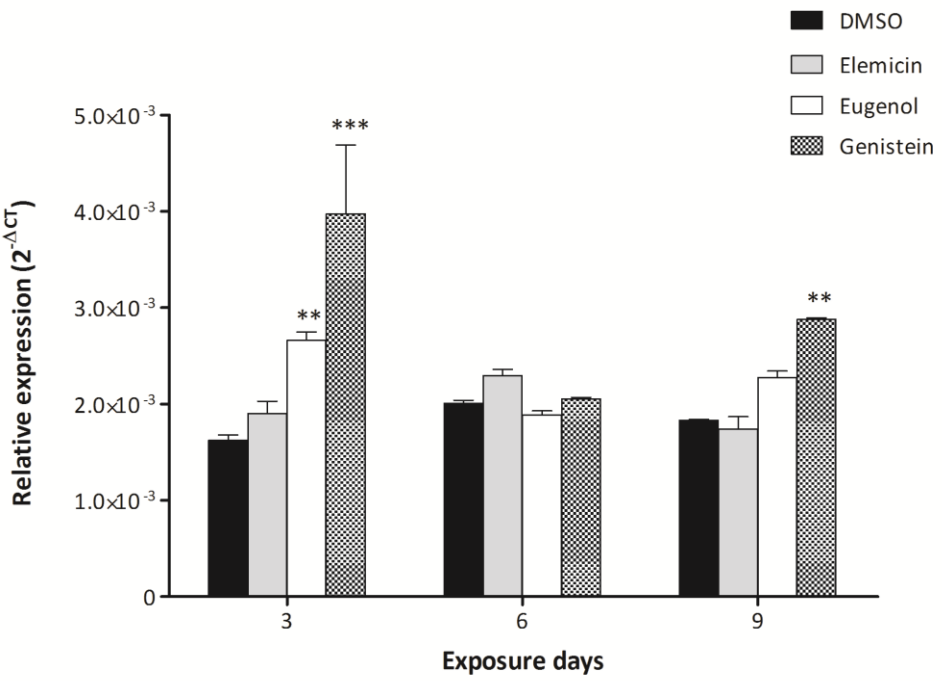

Figure 6.12 RASSF1A gene expression measured by RT-PCR. $^{**}P < 0.01$, $^{***}P < 0.001$

6.4 DISCUSSION

Diet contains the most variable and inevitable group of xenobiotics to which humans are exposed, yet it is the most difficult group of agents for which we can deduce a clear relation between exposure and disease (Doll and Peto, 1981). Studies have been suggesting that diet, maintaining a healthy weight and taking regular physical activity can prevent most common cancers. In fact, the ingestion of vegetables and fruit is considered protective (World Cancer Research Fund and American Institute for Cancer Research, 2007). According to some epidemiological studies and dietary interventions in animal models, it has been strongly suggested that nutrition-derived epigenetic modifications influence the epigenome and even cancer risk (Busch et al., 2015).

Several studies have demonstrated a link between early-life environmental conditions, namely nutritional imbalances and epigenetic events, and highlighted the developmental origins of health and disease. A striking example is given by the Dutch Hunger Winter Cohort (http://www.hongerwinter.nl/). Exposure to famine during gestation was associated with glucose intolerance, coronary heart disease, increased stress response and more obesity, and

women exposed to famine in early gestation had an increased risk for breast cancer (Roseboom et al., 2006). Moreover, individuals who were prenatally exposed to famine during the Dutch Hunger Winter in the years 1944 to 1945 had, 6 decades later, less DNA methylation of the imprinted *IGF2* gene compared with their unexposed, same-sex siblings (Heijmans et al., 2008). Insulin-like growth factor II (*IGF2*) is considered one of the best-characterized epigenetically regulated loci, and this gene is a key factor in human growth and development and is maternally imprinted. Imprinting is maintained through the *IGF2* differentially methylated region (DMR), the hypomethylation of which leads to bi-allelic expression of IGF2 (Heijmans et al., 2008). Although the exact mechanisms of the epigenetic alterations observed have not been elucidated, one possible explanation in this case is lack of dietary constituents that could modulate DNA methylation patterns.

In fact several pre-clinical studies have indicated a potential to modulate DNA methylation, mostly with polyphenols, namely the catechins found in abundance in green tea, which have been shown to reduce the risk of various diseases, including cancer. Epicatechin, epicatechin-3-gallate, epigallocatechin, and epigalocatechin-3-gallate (EGCG) are four major catechins present in green tea; EGCG constitutes more than 50% of the total catechin isolated from green tea and has been widely studied for its anticancer effects. Green tea polyphenols have been shown to exert anticancer effects by a number of different mechanisms, including the inhibition of cellular proliferation by induction of cell cycle arrest and apoptosis, and the suppression of oxidative stress, angiogenesis, invasion, and metastasis (Siddiqui et al., 2006), presumably through epigenetic regulation.

In the present study, as a proof of concept, we analysed methylation alterations in several genes induced by prolonged exposure (15 days) to a low dose (10 μM) of the alkenylbenzenes eugenol and elemicin or by the isoflavone genistein, in MCF-7 cells. The genes analysed have been reported to be involved in important biological pathways and human disease. Of the genes assessed, two of them, GSTP1 and RASSF1A, stand out for presenting normally with aberrant CpG methylation in numerous cancers. GSTP1A is a member of the 3 GST families of glutathione-S-transferases, which detoxify electrophilic xenobiotics, such as chemical carcinogens, environmental pollutants, and antitumor agents, and also inactivate endogenous alpha, beta-

unsaturated aldehydes, quinones, epoxides, and hydroperoxides formed as secondary metabolites during oxidative stress. These enzymes are also intimately involved in the biosynthesis of leukotrienes, prostaglandins, testosterone, and progesterone, as well as the degradation of tyrosine (Hayes et al., 2005). Hypermethylation of regulatory sequences at the detoxifying *GSTP1*3 gene locus is found in the majority (>90%) of primary prostate carcinomas but not in normal prostatic tissue or other normal tissues nor in benign hyperplasia of the prostate (Lee et al., 1994), being the most common genetic modification described thus far in prostate cancer (Cairns et al., 2001).

Likewise RASSF1A is also frequently hypermethylated in prostate cancer (Liu et al., 2002). RASSF1A is one of the most frequently hypermethylated genes so far described in human cancer. The CpG island promoter region of this gene is also highly methylated in several human cancers, most notably in small cell lung cancer, breast cancer, and renal cell carcinoma (Hesson et al., 2007). This may indicate that RASSF1A could play an important role as a cancer suppressor gene. We showed that eugenol and elemicin can increase expression of RASSF1 in MCF-7 breast cancer cells after prolonged exposure, as measured by RT-PCR, although MSP analysis was not clear cut. These discrepancies could be due to the CpG islands analysed. Many genes have more than one CpG island and methylation of CpG islands can vary. The miR124-1-170 promoter also underwent demethylation after 6 days exposure. Regarding LET7A3 at 3 days of exposure we can observe an increase in demethylation induced by all compounds, but only eugenol maintains unmethylation up to 15 days exposure. Our data are preliminary and thus should be followed up with further studies with different genes and miRNAs.

The use of phytochemicals as CAM in cancer patients is an area of serious concern because natural compounds, such as herbs and teas which contain numerous phytochemicals, may interact with cancer treatments. Furthermore, there is the common assumption that ‘‘natural’’ means safe; with the abundance of plants with known toxicity, this assumption is clearly ill founded (Ulbricht and Chao, 2010). Preparations of herbs and supplements may vary from manufacturer to manufacturer, and even between different batches from the same manufacturer. Because the active components of herbal products are often not identified, standardization may not be possible, and the clinical effects of different brands may not be

comparable. There may also be harmful contaminants in products not manufactured under good manufacturing practice (GMP) (Ulbricht and Chao, 2010). Thus, the long-term use of concentrated natural products is causing an increased concern. For example, a recent report evaluating the risk for end-stage renal disease in Taiwan found that the chronic use of traditional Chinese medicine herbs carried an increased risk of kidney failure (Ulbricht and Chao, 2010). In accordance with this view, the fact that we observed increased phosphorylation of ATM and H2AX after prolonged exposure to eugenol and elemicin indicate an increased risk of genotoxic effect. Most studies in toxicology have analysed the exposure of high doses of xenobiotics for short periods of time and his practice does not reveal the biological effects, namely epigenetic and genotoxic effects, after a prolonged exposure, more in accordance with chronic exposure to dietary components.

In conclusion our knowledge regarding nutritional and diet based epigenetics is still limited. In particular the effects of nutrients or bioactive food components on DNA methylation and gene expression is still not fully clear. Further studies are required, in particular whole genome methylation analysis after prolonged exposure to phytochemicals, coupled with whole genome expression analysis, in order to obtain an improved view of these effects.

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7. Chapter
DISCUSSION

The notion that natural extracts and plant products are safe has led to an increased consumption of phytochemicals in functional foods, food supplements and in CAM (Aggarwal et al., 2008; Aggarwal and Shishodia, 2006; Anand et al., 2008a; Anand et al., 2008b; Bakkali et al., 2008; Rietjens et al., 2008; van den Berg et al., 2011). Dietary components, including flavourings and additives should thus be properly studied to improve risk evaluation. Some flavourings, namely alkenylbenzenes, such as safrole, methyleugenol and estragole, are present in spices, essential oils and teas and are known for their genotoxic and carcinogenic properties. The EU Scientific Committee on Food (EU-SCF) has thus classified them with restrictions on their use (EU-SCF, 2001a, b, 2002). Safrole is classified by the International Agency for Research on Cancer (IARC) as a class 2B carcinogen (IARC, 1987). Other alkenylbenzenes, on the other hand, such as myristicin and eugenol, have been less studied and thus less data is available on their genotoxic or carcinogenic properties. About 30 alkenylbenzenes and a number of closely related compounds have been found in the essential oils of various plants (Miller et al., 1983) and two different classes of alkenylbenzenes have been identified: alkenylbenzenes with a 2,3-double bond such as estragole, eugenol, safrole, myristicin and elemicin, and propenylbenzenes with a 1,2-double bond such as trans-anethole and β-asarone (Lopez et al., 2015). Myristicin and eugenol are found in basil, anise, cinnamon, clove, fennel, nutmeg, parsley, star anise (Bakkali et al., 2008; NTP, 1983) and in some essential oils of clove, marjoram, bay leaf and cinnamon leaf (NTP, 1983; Slamenova et al., 2009). They are used as fragrances in the cosmetic and pesticide industry and as flavouring agents. Eugenol is widely used in dentistry as a cement material with zinc oxide or as a sedative agent (Hikiba et al., 2005; Slamenova et al., 2009). Myristicin is also used in traditional medicine to treat rheumatism, cholera, psychosis, stomach cramps, nausea, diarrhea, flatulence, and anxiety (Barceloux, 2008). In some countries herbal tea beverages, on the basis of bitter fennel fruits or bitter fennel oil, are used for babies and infants for carminative purposes (EFSA, 2009).

As such, the natural sources containing these molecules have been used not only in foods but in medicines for thousands of years (Sangster et al., 1987), both in essential oils, infusions, teas and in pharmaceutical formulations. Consequently, they are present and widely used not only in Western diets (van den Berg et al., 2011) but in medicines providing a significant potential risk

for human exposure to alkenylbenzenes, as some of these compounds have toxic, namely genotoxic, properties, as we showed over this thesis. International regulatory agencies have called for more data on the mechanisms of toxicity of these alkenylbenzenes in order to perform a better risk assessment, and thus, following out interest on natural compounds, we evaluated the mechanisms of genotoxicity of eugenol, estragole and myristicin

In Chapter 2 we evaluated the genotoxicity of eugenol in V79 cells using SCE and CAs assays, with and without rat liver biotransformation. Eugenol induced CAs, with significant increases (6.5% aberrant cells) albeit at a high dose, 1000 μM, in the presence of S9 mix, with a high frequency of chromatid exchanges. In particular, an increase of endoreduplicated cells was observed, suggesting that it may be a topoisomerase inhibitor. Endoreplication has been linked with DNA repair deficiencies, thus, although the doses used were high, in cases where cells are exposed to high doses *in vivo*, as with dentistry scenarios, a genotoxic effect may occur. In Chapter 3 we evaluated the genotoxicity of estragole in V79 cells using different cytogenetic endpoints and observed an increase in SCE without an exogenous biotransformation system and a decrease in its presence. Positive results were also observed in the alkaline comet assay without S9, indicating DNA strand breakage. Furthermore, a dose-dependent formation of DNA adducts in V79 cells was observed by the ^{32}P-postlabelling assay, which may be responsible for its genotoxicity. In Chapter 4 we compare the direct genotoxicity, repair and apoptotic activities of eugenol and myristicin in mammalian cells namely in DNA repair-proficient AA8 Chinese hamster cells, as well as repair-deficient (XRCC1^{-}) EM9 cells. Our positive results with the γ-H2AX assay for eugenol are in accordance with our previous results with the CA assay (chapter 2), showing that eugenol can induce DSBs. We also showed that myristicn was a greater inducer of apoptosis than eugenol, without being genotoxic. Chapter 5 provides further detail on the molecular mechanisms underlying the biological activity of myristicin. Myristicin can induce apoptosis, through alterations in the mitochondrial membrane potential, cytochrome c release, caspase-3 activation, PARP-cleavage and DNA fragmentation. The gene expression profile revealed an overall down regulation of DNA damage response genes after exposure to myristicin, with significant under-expression of genes associated with nucleotide excision repair, double strand break repair and DNA damage signalling and stress response. Finally in Chapter 6, as a proof of

concept, we analysed methylation alterations in several genes induced by prolonged exposure to a low dose (10µM) of the alkenylbenzenes eugenol and elemicin in MCF-7 cells. We showed that eugenol and elemicin can increase expression of RASSF1 in MCF-7 breast cancer cells after prolonged exposure, as measured by RT-PCR, although MSP analysis was not clear cut. Unexpectedly, we observed increased phosphorylation of ATM and H2AX after prolonged exposure to eugenol and elemicin, indicating an increased risk of genotoxic effects. Our knowledge regarding nutritional and diet based epigenetics is still limited.

In particular the effects of nutrients or bioactive food components on DNA methylation and gene expression is still not fully clear. Thus further studies are required, in particular whole genome methylation analysis and genotoxic effects after prolonged exposure to phytochemicals at low doses, coupled with whole genome expression analysis, in order to obtain an improved view of these effects. These studies are in line with the new strategies being pursued using genomics and proteomics to reveal biomarkers of exposure to low doses of chemicals.

Globally, our results may contribute to a further understanding of the potential risk of increasing our consumption of these alkenylbenzenes present in the diet, as was recommended by the JECFA meeting in 2008 (JECFA, 2008).

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ANNEX I - REGULAMENTARY AND EVALUATON AUTHORITIES

EFSA (European Food Safety Authority), receives requests for scientific advice manly from the European Commission, but also from the European Parliament or Member States. EFSA evaluates the safety of regulated food ingredients before they can be authorised for use on the European market. Food ingredients are chemical substances which are used as food additives, food enzymes, flavourings, smoke flavourings and sources of vitamins and minerals added to food. EFSA assesses the safety of new substances and of new proposed uses for currently authorised substances.

EFSA developed an approach for risk assessment of substances, which are both genotoxic and carcinogenic, called “Margin of Exposure” (**MOE**) (EFSA, 2005). The MOE approach uses a reference point, usually taken from data from an animal experiment that represents a dose causing a low but measurable cancer response. It can be for example the **BMDL10**, the lower confidence bound of the Benchmark Dose that gives 10 % (extra) cancer incidence (BMD10). The MOE is defined as the ratio between this reference point, the BMDL10, and the estimated dietary intake (**EDI**) in humans. EFSA has already clarified that the reference point is to be compared to various dietary intakes estimates in humans, taking into account different consumption patterns (EFSA, 2005)(EFSA 2009).

EMA (European Medicines Agency), have a Committee on Herbal Medicinal Products (**HMPC**). HMPC is the committee that is responsible for preparing the Agency's opinions on herbal medicines. The HMPC's activities aim at assisting the harmonisation of procedures and provisions concerning herbal medicinal products laid down in EU Member States, and further integrating herbal medicinal products in the European regulatory framework.

NTP (National Toxicology Program), is an interagency program whose mission is to evaluate agents of public health concern in the US by developing and applying tools of modern toxicology and molecular biology.

IARC (International Agency for Research on Cancer), is the specialized cancer agency of the World Health Organization (WHO). The IARC Monographs Programme is a core element of the Agency's portfolio of activities, with international expert working groups evaluating the evidence of the carcinogenicity of specific exposures. The close working relationship between IARC and its parent organization, **WHO**, allows the research findings of the Agency to be translated effectively into timely policies for cancer control. This is manifest, for example, in co-operation in terms of reduction in tobacco use, implementation of vaccination against viruses associated with cancer causation, or in assessing the effectiveness of intervention strategies. IARC is not involved directly in implementation of control measures, nor does it conduct research on treatment or care of cancer patients

The **IARC Monographs on the Evaluation of Carcinogenic Risks to Humans** identify environmental factors that can increase the risk of human cancer. Interdisciplinary Working Groups of expert scientists review the published studies and evaluate the weight of the evidence. Since 1971, more than 900 agents have been evaluated.

1	**2**	**3**	**4**
Group 1	**Carcinogenic** to humans	*Sufficient evidence in humans or sufficient evidence in animals and strong mechanistic data in humans*	118
Group 2A	**Probably** carcinogenic to humans	*Limited evidence in humans and sufficient evidence in animals*	79
Group 2B	**Possibly** carcinogenic to humans	*Limited evidence in humans and less than sufficient evidence in animals*	290
Group 3	**Not classifiable** as to its carcinogenicity to humans	*Inadequate in humans and inadequate or limited in animals*	501
Group 4	Probably **not carcinogenic** to humans	*Lack of carcinogenicity in humans and in animals*	1

1 Classification groups
2 Denomination
3 Evidence
4 Number of compounds already classified

JECFA (Joint FAO/WHO Expert Committee on Food Additives), is an international expert scientific committee that is administered jointly by the Food and Agriculture Organization of the United Nations (**FAO**) and the World Health Organization (**WHO**).

FDA (Food and Drug Administration), is a U.S.A. agency within the Department of Health and Human Services. The FDA's organization consists of the Office of the Commissioner and four directorates overseeing the core functions of the agency: Medical Products and Tobacco, Foods, Global Regulatory Operations and Policy, and Operations.

FEMA (Expert panel of the flavor and extract manufacturers association). FEMA is comprised of flavor manufacturers, flavor users, flavor ingredient suppliers, and others with an interest in the U.S. flavor industry. Founded in 1909, it is the national association of the U.S. flavor industry. FEMA works with legislators and regulators to assure that the needs of members and consumers are continuously addressed. FEMA is committed to assuring a substantial supply of safe flavoring substances. The Expert Panel of FEMA is the primary body for the safety evaluation of food flavoring for the flavor industry and the public through its "generally recognized as safe" (**GRAS**) assessment of flavoring substances. FEMA GRAS is the most widely recognized and admired industry GRAS assessment program.