

## Chapter 9

# Genotoxicity and Carcinogenicity of Herbal Products

Mélanie Poivre, Amandine Nachtergaele, Valérien Bunel, Okusa Ndjolo Philippe, and Pierre Duez

**Abstract** In 2012, the World Health Organization (WHO) recorded 14 million new cases of cancer and 8.2 million cancer-related deaths. Remarkably, the WHO estimates that 30% of cancer mortalities are due to lifestyle choices and environmental factors that can and should be avoided. In line with these recommendations, this chapter discusses the genotoxicity and carcinogenicity of herbal products. Although often perceived as innocuous by the general public, many herbs harbor phytochemicals that are either directly reactive towards DNA or likely to disturb cellular homeostasis, cell cycle, and/or genome maintenance mechanisms; this may translate into genotoxicity, carcinogenicity, or co-carcinogenicity. Genotoxicity refers to the deleterious effect of a chemical compound or a physical event on the genetic material; such genotoxic events are considered hallmarks of cancer risk. Nevertheless, much of the damage to the genetic material can be efficiently bypassed and/or repaired by the numerous genome maintenance mechanisms of the cell and may not lead to cancer. The long-term safety evaluation is probably better investigated through carcinogenicity, which denotes the capacity of a chemical substance or a mixture of chemical substances to induce cancer or increase its incidence. The major mechanisms of carcinogenicity are discussed along with biomarkers and approved regulatory guidelines. The recent development of innovative carcinogenicity testing strategies, especially based on functional genomics, are debated and evaluated for possible application to the precocious evaluation of herbal products'

---

M. Poivre • A. Nachtergaele • O. Ndjolo Philippe • P. Duez (✉)  
Unit of Therapeutic Chemistry and Pharmacognosy, Faculty of Medicine and Pharmacy,  
Research Institute for Health Sciences and Technology, University of Mons – UMONS,  
Mons, Belgium  
e-mail: [Pierre.DUEZ@umons.ac.be](mailto:Pierre.DUEZ@umons.ac.be)

V. Bunel  
Unit of Therapeutic Chemistry and Pharmacognosy, Faculty of Medicine and Pharmacy,  
Research Institute for Health Sciences and Technology, University of Mons – UMONS,  
Mons, Belgium

Laboratory of Pharmacognosy, Bromatology and Human Nutrition, Faculty of Pharmacy,  
Université Libre de Bruxelles – ULB, Brussels, Belgium

long-term safety. Finally, this chapter provides some examples of proven or suspected carcinogenic herbal products reported in the current literature.

**Keywords** Herbal products • Medicinal plants • Natural products • Genotoxicity • Carcinogenicity

## Abbreviations

2YRB	2-year rodent bioassay
AA	Aristolochic acid
AAN	Aristolochic acid nephropathy
BER	Base excision repair
CSC	Cancer stem cells
ECVAM	European Centre for the Validation of Alternative Methods
EMA	European Medicines Agency
FDA	Food and Drug Administration
HMP	Herbal medicinal products
HMPC	Committee on Herbal Medicinal Products
IARC	International Agency for Research on Cancer
ICH	International Conference on Harmonization
NER	Nucleotide excision repair
NOAEL	No observed adverse effect level
OECD	Organization for Economic Co-operation and Development
PA	Pyrrolizidine alkaloid
PFS	Plant food supplements
SAR	Structure-activity relationship
TCM	Traditional Chinese medicine
TFT	Trifluorothymidine
WHO	World Health Organization

## Introduction

Reported cases of cancer and cancer-related deaths are increasing worldwide, partly due to increased longevity and higher diagnosis rate. In 2012, the World Health Organization (WHO) recorded 14 million new cases of cancer and 8.2 million cancer-related deaths. Remarkably, the WHO estimates that 30% of cancer mortalities are due to lifestyle choices and environmental factors that can and should be avoided. Medicinal herbs are widely used throughout the world, both as primary healthcare solutions – mainly in developing countries – and as complementary or alternative medicines; their use has been continuously increasing for two decades in Western countries (Cheng and Leung 2012). The total market value of medicinal herbs accounts for about US \$83 billion and is expected to reach US \$107 billion by

2017 (Nutraceuticals 2012). This resurgence of interest in plant-based treatments seems to have various origins: it may come from patients' disappointment with standard treatments (in terms of efficacy and/or safety), from the rewarding feeling of active participation in the choice of therapeutic means, from the beliefs that the use of herbs is associated with a healthier lifestyle, and that herbal medicines, being "natural", are therefore harmless (Ekor 2014). Despite this positive perception of herbal treatments, their effectiveness and safety has most often not been evaluated per modern standards (Cheng and Leung 2012; Pelkonen et al. 2014); their quality, often unchecked, may be precarious, and cases of contamination, adulteration, toxicity, or poisoning are regularly detected (Vanherweghem et al. 1993; Liu et al. 2014). Until now, only a few quality toxicological studies have been carried out on the most widely used herbs; it is estimated that toxicological data are still missing for up to 90% of traditional Chinese herbal medicines (Cheng and Leung 2012), and the situation appears even worse for herbs used in developing countries, notably in African traditional medicine (Kahumba et al. 2015).

This chapter attempts to outline the issues of genotoxicity and carcinogenicity of herbal products. Indeed, many herbs harbor phytochemicals that are either directly reactive towards DNA, or are likely to disturb cellular homeostasis, cell cycle, and/or genome maintenance mechanisms, which may lead to genotoxicity, carcinogenicity, or co-carcinogenicity.

## ***Genotoxicity***

Genotoxicity describes the ability of chemical compounds and their metabolites to interact with DNA and/or the cellular machinery controlling the genome integrity (Butterworth 2006). Genotoxicants interact either directly with DNA or chromosomes to produce DNA damage such as adducts, strand breaks, chromosome breakages, etc., or indirectly, disturbing the genomic integrity through several mechanisms, notably by interaction (1) with proteins involved in DNA replication, transcription, or repair; (2) with components of mitotic spindle; or (3) with protein kinases in charge of cell cycle checkpoints (Magdolenova et al. 2014). Genotoxicants are usually classified according to their mutagenicity, through transformation of a DNA damage into a mutation, clastogenicity, through modification of chromosome structure, and aneugenicity, through changes in the number of chromosomes (loss or gain) (Muller et al. 2008; Botta 2013).

## ***Mutagenicity***

Mutagenesis, the process by which mutations appear, can arise spontaneously, without exposition to a mutagen (Smith 1992), or it can be induced by physical or chemical mutagens. The conversion of a DNA lesion into a permanent and heritable mutation requires DNA replication (Botta 2013), and so imbalances in the fidelity

of undamaged and damaged DNA replication appear as major causes of mutagenesis (Sarasin 2003; Loeb and Harris 2008). Introduced base substitutions can lead to “missense” and “nonsense” mutations, whereas insertions or deletions can induce frameshift mutations, both leading to altered gene expression (Magdolenova et al. 2014). Moreover, some non-genotoxic agents are able to increase mutagenesis indirectly by acting on DNA repair mechanisms or by stimulating cell proliferation, which increases the replication frequency (Dixon and Kopras 2004). Inorganic arsenic is an example of non-genotoxic mutagens; although negative in most mutagenic activity tests, exposure to arsenite (arsenic oxoanion where arsenic has an oxidation state of +3) has been strongly associated with an increased risk of skin, bladder, lung, and liver cancers. The mutagenesis of arsenic lies in its ability to increase the mutagenic activity of carcinogenic agents, such as UV irradiations, by interfering with DNA repair mechanisms of base excision repair (BER) and nucleotide excision repair (NER), through several mechanisms not completely elucidated (Shen et al. 2013; Andrew et al. 2006; Hubaux et al. 2013; Rossman 2003).

## ***Carcinogenicity***

Carcinogenesis is a complex process that is subject to intensive research. The following sections give insight into the various mechanisms involved in the genesis of cancer and point out the major critical events in which carcinogenicity can arise.

Carcinogens are substances able to “induce tumor (benign or malignant), increase the incidence or reduce the delay time of a tumor after their penetration into the body through inhalation, injection, dermal contact or ingestion” (Mulware 2012). The Organisation for Economic Co-operation and Development (OECD) classifies carcinogens into two categories: genotoxic carcinogens that initiate carcinogenesis by direct interaction with DNA and that are easily characterized by genotoxicity assays; and non-genotoxic carcinogens causing structural and functional DNA alterations that result in altered gene expression or signal transduction (Mulware 2012) and that are generally negative in genotoxicity assays (OECD 2007).

## **Genotoxic Carcinogens and the Somatic Mutation Theory**

In 1914, Theodor Boveri observed a link between genotoxicity, mutagenesis, and carcinogenesis. Accordingly, he concluded that “tumor growth is a consequence of incorrect chromosomal combination transmittable to daughter cells” (Balmain 2001). This discovery was the cornerstone of the somatic mutation theory that led to the development of *in vitro* screening assays for mutagenic compounds, notably the Ames test (Ames et al. 1973). In this theory, carcinogenesis is defined as a multi-step process starting with initiation, in which genomic alterations occur through chemical, physical, or biological (pathogens) agents. Initiated cells with selective growth advantage install as transformed clones during the promotion step. Finally,

progression is characterized by the transformation of preneoplastic lesions into clinically relevant cancer, with an increase of the metastatic potential and angiogenesis (Botta 2013; Loeb and Harris 2008; Monier 2008). This classical view of carcinogenesis is considered to be a simplification and cannot account for the various deregulated biological processes involved in cancer (Loeb and Harris 2008).

### The Mutator Phenotype

In their paper “The Hallmarks of Cancer” published in 2000, D. Hanahan and R.A. Weinberg described the molecular, structural, and behavioral capacities of cancer cells (Hanahan and Weinberg 2000) as (1) sustaining proliferative signaling; (2) evading growth suppressors; (3) resisting cell death; (4) enabling replicative immortality; (5) inducing angiogenesis; and (6) activating invasion and metastasis. Recent advances in carcinogenesis led the authors to add two emerging characteristics to this list: (7) reprogramming of energy metabolism to support growth and continuous cell proliferation; and (8) evading immune destruction. According to the authors, these characteristics are underpinned by the genomic instability and inflammatory status of pre-malignant and malignant lesions (Hanahan and Weinberg 2011).

### Mutator Phenotype and Genomic Instability

The discovery of some critical mutated genes in many cancers (Davies et al. 2002) has contributed to build the hypothesis that alterations in some specific genes are responsible for tumor initiation, maintenance, and progression (Quante and Wang 2008). A malignant transformation would require a number of independent mutations (Knudson 2001): (1) oncogene activation; (2) tumor suppressor gene inactivation; and (3) telomerase constitutive expression (e.g., hTERT) (Botta 2013; Dixon and Koprass 2004). Oncogenes are genes coding for growth factors (e.g., PDGF), tyrosine kinase surface receptors (e.g., EGFR, HER), anti-apoptotic proteins (e.g., BCL-2) (Martinez-Arribas et al. 2007), nuclear transcription factors (e.g., MYC), or signal transducing G-proteins (e.g., RAS) (Hesketh 1997), and are involved in signalization pathways that stimulate cell proliferation. These genes are mainly active during embryogenesis but can be activated during adulthood through mutation or chromosomal rearrangement. Tumor suppressor genes code for proteins associated with cell cycle arrest, apoptosis and DNA repair. These genes are classified either as “gate keepers” coding for proteins involved in the control and regulation of cell proliferation (e.g., P53, RB1, APC) or as “care takers” coding for proteins involved in the genome repair and stabilization (e.g., BRCA1, BRCA2, MSH2, MLH1) (Botta 2013; Dixon and Koprass 2004). Regions of repetitive DNA sequences at each end of a chromosome (telomeres) are synthesized by an enzyme called telomerase (hTERT) that prevents their degradation. The gradual reducing of telomeres during each cell division is a normal process for the cell, ultimately leading to apoptotic death. To counter this, many tumor cells constitutively express telomerases (Dixon and Koprass 2004).

In 1974 L.A. Loeb described for the first time the mutator phenotype hypothesis (Loeb et al. 1974). He calculated that the mutation rate of non-cancerous cells is insufficient to generate the large number of mutations found in cancerous cells. According to this hypothesis, mutations in specific genes governing genomic stability (oncogenes and tumor suppressor genes) lead to an enhanced genomic instability that substantially increases the mutation rate and justifies the multiple mutations observed in cancer cells (Loeb and Harris 2008; Loeb 2011). However, it still remains unclear whether genomic instability is a prerequisite or a consequence of cancer development, and arguments have developed on both sides (Marx 2002). The high prevalence of cancers among patients with genetic diseases linked to defects in genes responsible for genetic stability (Cleaver 2005), the existence of a mutator phenotype in DNA repair proteins deficient cells (Friedberg et al. 2002), and the demonstration of a mutation rate 200 times higher in tumor tissues (Bielas et al. 2006) are strong arguments in favor of such a mutator phenotype. On the other hand, it has been shown that, in highly proliferative tissues, the rate of spontaneous mutations is enough to allow the accumulation of mutations and provide a selective advantage required for clonal expansion (Sarasin 2003; Dixon and Kopras 2004; Wang et al. 2002). According to these arguments, the genomic instability would take place later in cancer development to contribute to its expansion in the body (Marx 2002).

### **Non-genotoxic Carcinogens**

Non-genotoxic carcinogens exert various modes of action including (1) mitogen stimulation of growth through hormonal effects eventually mediated by a receptor (e.g., binding to estrogen receptor, disturbance of the synthesis or secretion of thyroid hormones by anti-thyroid substances); (2) promotion of tumors (modulation of DNA repair mechanisms and cell cycle control); (3) induction of a specific tissue toxicity and targeted inflammation, resulting in a regenerative hyperplasia; (4) immune suppression; (5) inhibition of intercellular communications through gap junctions, essential to cellular homeostasis; and (6) epigenetic modifications (Butterworth 2006; Hernandez et al. 2009). The great diversity in modes of action and tissue specificities of these agents, combined with their absence of genotoxicity, makes carcinogenicity prediction extremely challenging.

### **Stem Cells**

The origin of cancer is also attributed to stem cells, a sub-population of cells able to divide and generate numerous copies identical to themselves (self-renewing), and differentiated cell lineages (Gonzalez and Bernad 2012). In the middle of the nineteenth century, two German pathologists, Julius Cohnheim and Rudolph Virchow, observed similarities between embryonic and cancerous tissues and hypothesized that tumors would arise by reactivation of sleeping embryonic rest tissue (Virchow

1855; Cohnheim 1875). This “embryonic rest hypothesis of cancer” then postulates that adult cancers develop from stem cells. In this theory, a tumor is seen as an aberrant and heterogeneous organ in which only a small portion of cancer cells, the cancer stem cells (CSC), is able to initiate tumor growth, proliferate extensively, and present a metastatic potential (Quante and Wang 2008). Recent technological developments allowed isolating this cancer stem cells sub-population from the other tumor cells using specific surface markers (Monier 2008). The CSCs are capable, just as normal stem cells, of self-renewal, differentiation, and asymmetric division to generate both a new identical stem cell and a progenitor cell with a limited lifetime but responsible for the proliferation (Monier 2008). The injection to immunodeficient mice of a small number of CSCs, but not of other tumor cells, effectively leads to tumor development (O'Brien et al. 2007). The origin of these cancer stem cells is still a matter of debate and seems to vary from one tumor type to another (Hanahan and Weinberg 2011). In some tumors, the CSC would originate from a tissue stem cell that has undergone a cancerous transformation. In others, CSC would drift from progenitor cells arising from the asymmetric division of a normal tissue stem cell (Monier 2008; Hanahan and Weinberg 2011; Quante and Wang 2008). The recent discovery of circulating progenitor cells with stem cells lineage specific properties raised questions about the existence of distinct stem cell populations for each tissue or the existence of a centralized stem cell source (Quante and Wang 2008; Shaked et al. 2006).

### **Influence of Epigenetics**

It is nowadays acknowledged that the altered expression of oncogenes and tumor suppressor genes can also arise from epigenetic modifications; these involve DNA chemical modifications free of sequence alteration, such as nucleotide methylation, histone modification (acetylation, methylation and phosphorylation), chromatin remodeling, nucleosome positioning, and non-coding RNA modulation (e.g., microRNA) (Dixon and Koprass 2004; Vineis et al. 2010; Migheli and Migliore 2014). Genetic and epigenetic factors interact and influence themselves during carcinogenesis, and to date, no cancer has been detected with only a genetic or epigenetic background (Migheli and Migliore 2014; Burgio and Migliore 2015).

### **Influence of Tumor Microenvironment**

Neglected for too long by the somatic mutation theory, the microenvironment of tumors plays a predominant role in carcinogenesis. This tumor microenvironment is defined as “the normal cells, molecules, and blood vessels that surround and feed a tumor cell. A tumor can change its microenvironment, and the microenvironment can affect how a tumor grows and spreads” (National Cancer Institute 2015). The information coming from the tumor environment induces dynamic mechanisms that yield phenotypic alterations, most probably through epigenetic modifications (Burgio and

Migliore 2015). During clonal expansion, the tumor microenvironment dictates selective conditions; a mutation on a specific gene can lead to clonal expansion if this mutation confers a selective advantage over normal cells towards the microenvironment (Sarasin 2003; Vineis et al. 2010; Wu and Starr 2014).

## **Cancer and Inflammation**

An inflammatory state of pre-malignant and malignant lesions favor tumor progression through various mechanisms (Hanahan and Weinberg 2011). Indeed, it has been shown that chronic inflammatory situations such as viral infections (human papilloma virus, hepatitis-B, etc.), obesity, chronic gastric reflux, chronic colitis, and Crohn's disease are associated with cancer development (Coussens and Werb 2002). The underlying mechanisms involve oxygen and nitrogen reactive species, inflammatory cytokines, prostaglandins, and microRNA produced during inflammation. The chronic production of such mediators will cause DNA damage, alter gene expression, and provoke cellular proliferation changes (Loeb and Harris 2008; Quante and Wang 2008; Vineis et al. 2010).

## **Mechanism of Malignancy Is Still a Matter of Huge Debate**

In January 2015, C. Tomasetti and B. Vogelstein investigated the significant variation in cancer risk between different types of tissues, showing an important correlation between the number of stem cell divisions in a particular tissue and the risk of cancer (Tomasetti and Vogelstein 2015). Based on this result, they showed that only a third of cancer risk variation from one tissue to another would be attributed to genetic predisposition and environmental factors. The majority of observed tissue-to-tissue variation would be due to "bad luck" arising from stochastic mutations during DNA replication in non-cancerous stem-cells. The bad luck hypothesis of cancer has been the subject of considerable criticism arguing, among others, that the authors have considered stem-cell division rates and extrinsic risk factors as entirely independent. In January 2016, Y.A. Hannun and his colleagues provided evidence that intrinsic risk factors contribute only modestly (less than 10–30% lifetime risk) to the mechanism of malignancy (Wu et al. 2016). The exact contribution of external and internal factors in cancer development is still open to debate and could have implications in cancer prevention strategies. Primary prevention (e.g., lifestyle modification, HPV vaccines) would impact on the risk of cancers triggered by environmental factors but would not be effective on cancers for which the risk is attributable to "bad luck" or internal factors. For these cancers, secondary prevention (early detection of cancer) and chemoprevention (dietary agents modulating DNA replication and/or repair (Charles et al. 2012, 2014; Nachtergaeel et al. 2013)) would be the most effective strategies to decrease cancer mortality.

## Genotoxicity and Carcinogenicity Assessment

There is currently no single validated test able to provide information on the three genotoxicity critical end-points, which are mutation induction, clastogenicity, and aneugenicity; a battery of tests is thus needed to determine the genotoxic and mutagenic potential of a compound. Moreover, due to the diversity of the end-points, genotoxicity and/or carcinogenicity cannot be assessed in a single assay (Maurici et al. 2004).

### Genotoxicity Assessment

Genotoxicity assays are dedicated to the detection of compounds that can induce genetic damage by various mechanisms (ICH 2014). The major challenge in genotoxicity testing resides in the development of methods that can reliably and sensibly detect either such a vast array of damages, or a general cellular response to genotoxic insult. It is recognized that no single test can detect every genotoxicant, and therefore the concept of the battery of tests has been implemented in many regulatory guidelines (Billintona et al. 2008).

Methods for genotoxicity assessment include *in silico* and structure alert methods; as well as *in vitro* and *in vivo* methods.

### In Silico and Structure Alert Methods

#### *In silico* methods

*In silico* methods aim at predicting biological activities of a molecule from its physicochemical properties (Combes 2012). These predictive methods generally rely on computational tools, mathematical calculation, and analysis of predicted or experimental data through computer-based models (Valerio 2009) that are generally classified as: (1) rule-based expert systems (e.g., DEREK), which estimate the presence of a DNA-reactive moiety in a given molecule (Greene 2002); (2) quantitative structure-activity relationship models, so-called “QSAR” models (e.g., TOPKAT) that use “electro-topological” descriptors rather than chemical structure to predict mutagenic reactivity with DNA; and (3) three-dimensional computational DNA-docking models to identify molecules that are capable of non-covalent DNA interaction.

- Benefits: *In silico* prediction systems have many advantages, such as their low-cost, rapidity, high reproducibility, low/no compound synthesis requirements, constant optimization, and potential to reduce or replace the use of animals (3R policy, aiming at replacing, reducing, and refining the use of animals (Fjodorova et al. 2010)).

- **Limitations:** The lack of factual toxicity data, inappropriate (simplistic) modeling of some endpoints, and poor domain applicability of models represent their main limitations. The application of *in silico* methods to complex mixtures such as herbal extracts is limited to the detection of known or new structural alerts for genotoxicity. However, they could help to elucidate which compounds are responsible for a proven effect (Valerio 2009; Ouedraogo et al. 2012).

### Structure alert methods

Structural alerts, also called “toxicophores”, are defined as molecules or moieties that are known to be associated with toxicity; their presence alerts the investigator to their potential toxicity. Well-characterized genotoxic compounds include (1) 1–2 unsaturated pyrrolizidine ester alkaloids from many *Boraginaceae*, *Asteraceae*, and *Fabaceae* (Fu et al. 2002; Chen et al. 2010; Xia et al. 2008); (2) aristolochic acids (AA), nitro-polyaromatic compounds notably responsible for terminal nephropathies observed upon intoxication by many *Aristolochia* species (Chan 2003; Mei et al. 2006); and (3) allylalkoxybenzenes, e.g., eugenol, methyleugenol, estragole, safrole (4-allyl-1,2-methylenedioxybenzene) or asarone, potentially genotoxic components from some essential oils. The notion of threshold for genotoxic insults is still a matter of serious debate; consequently, proved toxicophores should be proscribed from herbal medicines or at least severely limited (Ouedraogo et al. 2012).

### *In Vitro* Methods

The term *in vitro* (Latin for “in the glass”) refers to experiments carried out in a controlled environment, outside of a living organism. *In vitro* methods are based on the use of pro- or eukaryotic cells and tissue cultures (Brusick 1980). Increasingly, human cells are used since they better predict human toxicity (ECVAM 2015).

- **Benefits:** *In vitro* assays are relatively inexpensive, easy to conduct, and do not involve the use of animals. *In vitro* assays typically provide an initial indication of the genotoxicity of a chemical, and the results often guide eventual subsequent *in vivo* studies (OECD 2014).
- **Limitations:** A battery of tests is required to investigate the multiple aspects of genotoxicity. Oversensitivity and low specificity represent common problems, compared to *in vivo* situations. *In vitro* assays notably require supplementation with exogenous metabolic activation enzymes (e.g., S9 fraction of liver homogenate) in order to simulate mammalian metabolism. Moreover, *in vitro* testing on mammalian cells may use cell lines that are not relevant to predict genotoxic endpoints at target organs (Maurici et al. 2004; Ouedraogo et al. 2012; Brusick 1980). Effectively, the commonly used cell lines are often deficient in DNA repair, p53 function, or metabolic competency, and many derive from malignancies (Walmsley and Billinton 2011).

The emerging toxicogenomics area could lead to a better understanding of genotoxicity/mutagenicity processes and help in the development of more accurate *in vitro* models (Burgio and Migliore 2015; Hoet 2013). Nevertheless, to overcome

the limitations of *in vitro* testing and fully replace the use of animals, refined and validated toxicokinetics and metabolism-competent model systems are sorely needed to accurately predict or mirror the *in vivo* situation.

### ***In Vivo* Methods**

*In vivo* methods are generally recommended to complete the information gathered during *in vitro* investigations and/or to overcome their limitations (Ouedraogo et al. 2012). The term “*in vivo*” refers to experimentations based on a whole, living organism – as opposed to a partial or dead organism – and consist of either animal studies or clinical trials (ECVAM 2015). For evident ethical reasons, genotoxicity studies are only performed in animals.

- **Benefits:** *In vivo* studies include pharmacokinetic factors that are able to influence the outcomes of toxicity assessment, which allows better extrapolation of potential noxious effects to humans. The number of test animals, gender, dosage, time, and the use of suitable controls are important parameters to consider (Ouedraogo et al. 2012; Hartung 2011).
- **Limitations:** *In vivo* tests contradict the 3R philosophy, and entitle chronic studies with longer durations and costs. Moreover, the metabolism of drugs can vastly differ among mammals, so both negative and positive data may not be transferable to humans. *In vivo* tests, such as the bone marrow micronucleus test, are relatively insensitive (Muller et al. 1999), so the established *in vitro* genotoxicity tests are still considered first-line tests, as they are sensitive enough to detect the great majority of genotoxins. Several tests (typically on bone marrow, blood, or liver) are deemed unable to provide additional useful information as compared to *in vitro* assays, especially for compounds with poor systemic absorption, e.g., radioimaging agents and aluminum-based antacids (ICH 2014; FDA 2012).

### ***Guidelines on the Genotoxicity Assessment***

The OECD, the Food and Drug Administration (FDA), the International Agency for Research on Cancer (IARC), the European Medicines Agency (EMA), and the European Centre for the Validation of Alternative Methods (ECVAM) are all organizations that investigate the validation of tests and provide a general framework, practical approaches, and rules for data interpretation (EMEA 2008a). Guidelines have been established by OECD, ICH, and EMA committees (ICH 2014; OECD 2014) to optimize genetic toxicology testing for the prediction of potential human risks. They also provide guidance on the interpretation of results, describing internationally acknowledged standards for follow-up testing and interpretation of positive *in vitro* and *in vivo* results, including the assessment of non-relevant findings (ICH 2014). The “Guideline on non-clinical documentation for herbal medicinal products in applications for marketing authorization (bibliographical and mixed applications) and in applications for simplified registration”, implemented by the EMA Committee

on Herbal Medicinal Products (HMPC), establishes a step-by-step procedure for assessing the genotoxicity of herbal medicinal products (HMPs) (EMA 2008b).

## Tests Approved for Genotoxicity Testing

Guidelines for the assessment of the genotoxicity of pharmaceuticals

The registration of pharmaceuticals requires a comprehensive assessment of their genotoxic potential. The recommended battery of tests is described in the ICH Harmonised Tripartite Guideline Report S2, “Guidance on genotoxicity testing and data interpretation for pharmaceuticals intended for human use”. The recommended battery includes a bacterial reverse mutation test (Ames test), which effectively detects genetic changes and the majority of carcinogens genotoxic to rodents and humans, and mammalian *in vitro* and/or *in vivo* tests, mandatory or decided on obtained data (ICH 2014). Three *in vitro* mammalian assays are widely used, considered sufficiently validated, equally appropriate, and therefore interchangeable for the measurement of chromosomal damage when used together with other genotoxicity tests: (1) the *in vitro* metaphase chromosome aberration assay; (2) the *in vitro* micronucleus assay; and (3) the mouse lymphoma L5178Y cell thymidine kinase gene mutation assay. *In vivo* assays are included in the test battery to help identify false negatives (e.g., agents mutagenic *in vivo* but not *in vitro*), and to provide insights on the influence of pharmacokinetics.

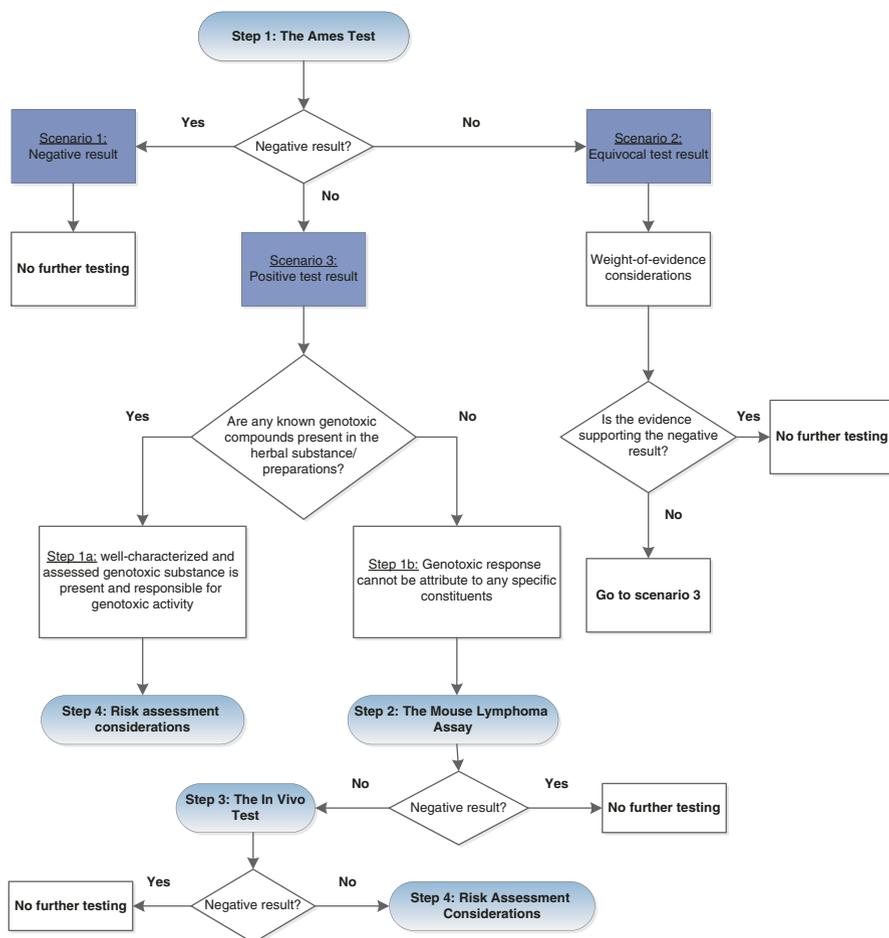
Guidelines for the assessment of the genotoxicity of herbal medicinal products

HMPs present a number of characteristics that differentiate them from other medicinal products, explaining the need for specific guidance. Herbal products are complex mixtures containing a large number of constituents that are sometimes present in highly variable amounts. The complete composition of a preparation is often unknown, and thus structural alerts for toxicants can be unraveled; moreover, the composition may vary with many parameters (harvesting time, geographical origin, mode of preparation, contamination, adulteration) that could invalidate previously obtained genotoxicity data. Nevertheless, HMPs are framed by similar regulations as for other medicinal products for human use; as with other medicinal products, signals of adverse effects could arise occasionally through pharmacovigilance (EMA 2008a).

The HMPC stepwise testing process for HMPs involves a battery of genotoxicity tests, as described in a decision tree (Fig. 9.1 The stepwise testing process of herbal medicinal products, adapted from EMA (FDA 2012)).

### Step 1: The Ames test

The Ames test, a bacterial reverse gene mutation test, should be performed and interpreted in conformity with existing OECD and EU guidelines. Briefly, a set of mutated *Salmonella typhimurium* strains, each auxotroph for a specific amino acid, is incubated under selected pressure (low level of the specific amino acid), and in the presence of the studied substance/preparation, with or without a metabolic activation system. Mutations occurring in the non-functional gene will restore the capability of bacteria to synthesize the specific amino acid (“revertants”). The number of revertants correlates quite well with the mutagenic potential of a substance (ICH 2014; Ouedraogo et al. 2012; OECD 2014; Hoet 2013; EMA 2008a).



**Fig. 9.1** The stepwise testing process of herbal medicinal products (Adapted from EMA (2008a))

- In the case of a negative result, no further genotoxicity testing is required.
- Equivocal test results require special considerations; a repetition of the experiment should generally be envisaged.
- In case of positive results:
  - the presence of acknowledged genotoxic compounds not known to be carcinogenic (e.g., quercetin) can tentatively explain the mutation.
  - the absence of such genotoxic compounds implies that the herbal product has to be studied in a Step 2 test.

#### Step 2: The mouse lymphoma assay or other mammalian cell assays

As for the Ames test, the mouse lymphoma assay should be performed and interpreted in conformity with existing OECD and EU guidelines. Briefly, L5178Y mouse lymphoma cells in culture are exposed to a compound or a preparation, and mutants in the thymidine kinase gene (mutation  $TK^{+/-} \rightarrow TK^{-/-}$ ) are detected by their resistance to the cytotoxic pyrimidine analogue trifluorothymidine

(OECD 2014). This assay may confirm or refute positive findings in the Ames test. Moreover, it may give information on the ability of herbal products to cause chromosomal damage. If other mammalian cell assays are used for genotoxicity tests, their use has to be justified (OECD 2014; Hoet 2013).

- In the case of a negative result, no further testing is required.
- In the case of a positive result, the relevance of the finding should be thoroughly assessed, as it is known that the mouse lymphoma assay is associated with false positives. If the test is unequivocally positive (gene mutation or chromosomal damage), it is advisable to proceed to step 3. If the herbal preparation is known to contain a compound with chromosomal damaging properties, it may be advisable to perform the *in vitro* micronucleus test in mammalian cells in culture (EMA 2008a).

#### Step 3: The rodent micronucleus test or other *in vivo* genotoxicity tests

The rodent micronucleus test should be performed and interpreted in conformity with the existing OECD and EU guidelines. Briefly, mice or rats are treated with the compound or preparation (in an appropriate vehicle and via appropriate route of administration). The proportion of micronuclei in bone marrow and/or peripheral blood cells can identify agents causing structural and numerical chromosomal changes (OECD 2014; EMA 2008a).

- In the case of a negative result, no further testing is required.
- In the case of a positive result, it is advisable to proceed to step 4.

#### Step 4: The risk assessment considerations

No single specific approach has been recommended for risk assessment (EMA 2008a), since many points have to be taken into consideration. A risk assessment through the “Threshold of Toxicological Concern” approach is possible whenever a herbal preparation contains an identifiable genotoxic compound that presents a demonstrated threshold mechanism; permissible exposure levels – without appreciable risk of genotoxicity – can be established according to the usual “No Observable Effects Level” (NOEL) method. However, as herbal preparations are complex mixtures with partially unidentified components, it is quite possible that the compound(s) responsible for genotoxicity is/are still not identified at the end of the testing protocol. Thus, the usual procedure for toxicity testing and risk assessment of mixtures should consist in isolating and identifying various major constituents and testing them individually (EMA 2008a) – which is a time-consuming, costly, and probably unrealistic approach for herbal medicines.

This HMPC stepwise testing process for herbal medicinal products effectively defines the Ames test as the primary endpoint which, if negative, accepts the drug as probably “non-genotoxic”. This is not entirely satisfying, however (Ouedraogo et al. 2012), and has been greatly debated (EMA 2008b); indeed (1) the Ames test does not detect every genotoxic insult; and (2) since some common

compounds, including flavonoids, yield very positive Ames tests but are not carcinogens, they may effectively mask the genotoxic effect of real carcinogens.

### **Novel Approaches for Genotoxicity Testing**

“Omics” studies involve a large number of measurements per endpoint to acquire comprehensive, integrated understanding of biology and to identify various factors simultaneously (e.g., genes, RNA, proteins and metabolites) rather than each of those individually. Toxicogenomics study the interactions between the structure and activity of the genome and the adverse biological effects of exogenous agents. The toxic effects of xenobiotics on biological systems are generally reflected at the cellular level by their impact on gene expression (transcriptomics), and on the production of proteins (proteomics) and small metabolites (metabonomics) (Ouedraogo et al. 2012; Borner et al. 2011). Genetic variation and expression signatures can be used to screen compounds for hazards, to assess cellular responses to various dosages, to classify toxicants on the basis of mechanisms of action, to monitor exposure of individuals to toxicants, and to predict individual variability in sensitivity to toxicants (NAP 2007). Toxicogenomics effectively allows understanding dose-response relationships, cross-species extrapolations, exposure quantification, underlying mechanisms of toxicity, and the basis of individual susceptibilities to particular compounds (NAP 2007). We recently reviewed novel approaches for genotoxicity testing, based on “omics” technologies, with their applications to herbal drugs, including their advantages and limitations (Ouedraogo et al. 2012).

### ***Carcinogenicity Assessment***

The assessment of carcinogenicity aims to identify a tumorigenic potential in animals and to evaluate the possible risk to humans (ICH 2015). Determining the carcinogenic potential is an important, complex, and imperfect exercise. The methods for such determinations are expensive and long, and they use many animals; moreover, the extrapolation of data from such studies to human risk is imprecise (Jacobson-Kram 2009).

### **Human Carcinogens Classification**

Carcinogenic substances induce tumors (benign or malignant), increase their incidence or malignancy, or shorten the time for tumor occurrence. As discussed in section “**Carcinogenicity**”, carcinogens are classified as either genotoxic or non-genotoxic, depending on their mode of action. They can also be classified as threshold-unlikely (DNA-reactive genotoxic compounds, for which NOAEL – no

observed adverse effect level – cannot be estimated) or threshold-likely (non-DNA reactive genotoxins and non-genotoxic carcinogens, for which NOAEL can be estimated) (Hernandez et al. 2009). The IARC classifies human carcinogens into five groups depending on their carcinogenic potential to humans (Hernandez et al. 2009; IARC 2015):

- *Group 1*: Carcinogens to humans (117 agents). This group includes 61 chemicals, 9 viruses or pathogens (e.g., HIV), 19 exposure circumstances (e.g., chimney sweeping), and 16 mixtures (e.g., coal-tars) including as herbal products: areca nut, plants containing aristolochic acid, betel quid with and without tobacco, tobacco (smokeless, smoking, and passive smoking), and aflatoxins (from contamination by producing organisms).
- *Group 2A*: Probable carcinogens to humans (74 agents), including 50 chemicals, 2 viruses or pathogens, 7 exposure circumstances, and 7 mixtures, including as herbal products: emissions from high-temperature frying (applied to some herbal medicines processing) and hot beverages.
- *Group 2B*: Possible carcinogens to humans (287 agents), including 224 chemicals, 4 viruses or pathogens, 7 exposure circumstances, and 13 mixtures, including as herbal products: *Aloe vera*, whole leaf extract, bracken fern, coffee (urinary bladder), *Ginkgo biloba* extract, goldenseal root powder, kava extract, and pickled vegetables (traditional Asian), toxins derived from *Fusarium moniliforme* (from contamination by producing organisms). Moreover, monocrotaline and safrole are also both found in this group.
- *Group 3*: Non classifiable carcinogens (503 agents), including 496 chemicals, 8 exposure circumstances, and 11 mixtures, including as herbal products: Madder root (*Rubia tinctorum* L.) and mate (*Ilex paraguariensis* A.St.-Hil.). Retrorsine and eugenol are also both classified in this group.
- *Group 4*: Probably not carcinogenic to humans. This group contains a single agent: caprolactam.

### **Guidelines for Carcinogenicity Assessment of Pharmaceuticals**

The strategy for testing the carcinogenic potential is developed according to the results of genetic and repeated-dose toxicology studies (EMA guidelines S2A and S2B), pharmacodynamics (selectivity, dose-response), and pharmacokinetics in animals and in humans (Guideline S1C), intended patient population, and clinical dosage regimen (guideline S1A) (ICH 2015). Nowadays, there is no carcinogenic assessment strategy recommended or required for HMPs. Guidelines for the carcinogenicity testing of pharmaceuticals could probably be extrapolated to them whenever deemed necessary; however, most of the time, the carcinogenic potential of an HMP is not evaluated and only genotoxicity data are used to “predict” carcinogenicity.

## Factors to Consider for Carcinogenicity Testing of Pharmaceuticals

### Duration and exposure

Carcinogenicity studies should be performed for any pharmaceutical for which expected clinical use is continuous for at least 6 months or repeated in an intermittent manner (e.g., for depression, anxiety, or allergy). Pharmaceuticals administered infrequently or for short durations of exposure (e.g., anesthetics and radiolabeled imaging agents) do not require carcinogenicity studies unless there is cause for concern (ICH 2015).

### Cause for concern

Carcinogenicity assays may be recommended for some pharmaceuticals if there is concern about their carcinogenic potential, which includes: (1) previous demonstration of carcinogenic potential in the product class considered; (2) structure-activity relationship suggesting carcinogenic risk; (3) evidence of preneoplastic lesions in repeated-dose toxicity studies; and (4) long-term tissue retention of parent compound or metabolite(s), resulting in local tissue reactions or other pathophysiological responses (ICH 2015).

### Genotoxicity

Unequivocally, genotoxic compounds (in the absence of other data) are presumed to be carcinogens and warrant long-term carcinogenicity studies (ICH 2015). In addition to their use as a screening tool, genotoxicity data constitute part of the weight of evidence when evaluating environmental chemicals and herbal medicines (HMPC strategy). In practice, environmental contaminants have not been regulated as carcinogens on the basis of positive genotoxicity results alone. Nonetheless, positive tests are generally indicative of chemicals capable of inducing cancer via a genotoxic or mutagenic activity (Guyton et al. 2009).

### Indication and patient population

Pharmaceuticals developed to treat life-threatening or severely debilitating diseases do not always require carcinogenicity testing before market approval; this is particularly the case for anti-cancer agents. These time-consuming studies can be conducted post-approval in order to speed the availability of the product on the market (ICH 2015).

### Route of exposure

If possible, the route of exposure in animals should be the same as the intended clinical routes. If similar metabolism and systemic exposure can be demonstrated by differing routes of administration, carcinogenicity studies should be conducted only by a single route (ICH 2015).

### Extent of systemic exposure

Pharmaceuticals applied topically (e.g., dermal and ocular routes of administration) may need carcinogenicity studies. However, pharmaceuticals showing poor systemic exposure from topical routes in humans may not need oral administration studies. Moreover, for various salts, acids, or drug bases, evidence of no

significant changes in pharmacokinetics, pharmacodynamics, or toxicity should be provided (ICH 2015).

### Tests Approved for Carcinogenicity Assessment

The assessment of the carcinogenic potential of pharmaceuticals usually involves two rodent species (2-year rodent bioassay – mostly rat and mouse) (ICH 2015; Raghava et al. 2014). The species are selected according to data on pharmacology, repeated-dose toxicology, metabolism, toxicokinetics, and route of administration. In the absence of a clear advantage favoring a species, rat models are recommended (ICH 2015).

Further mechanistic studies are often useful for the interpretation of carcinogenicity data and can provide a perspective on their relevance in humans; these may investigate (1) cellular changes in relevant tissues, using morphological, histochemical, or functional criteria, e.g., dose-relationship for apoptosis, cell proliferation, liver foci, alteration or changes in intercellular communication; and (2) biochemical measurements, e.g., plasma hormone levels, growth factors, binding proteins (i.e.,  $\alpha 2\mu$ -globulin) and tissue enzyme activity (ICH 2015). In some cases, additional genotoxicity testing in appropriate models may be required (Butterworth 2006); this would be the case of compounds resulting in negative outcomes in the standard test battery, but which demonstrated effects a carcinogenicity test with no clear evidence for an epigenetic mechanism. This additional testing can include modified conditions for metabolic activation in *in vitro* tests or can include *in vivo* tests measuring genotoxic damage in target organs (ICH 2015).

### Limitations of the 2YR Strategy

For each compound, the 2YR strategy requires more than 800 rodents with more than 40 histopathological tissue analyses for each of them. The cost of this approach can reach US \$2.4 million per compound, depending on the route of administration, number of doses, and the chemical to evaluate. Thus, in addition to denying the 3R policy, the 2YR strategy is costly and time-consuming (Fjodorova et al. 2010; Raghava et al. 2014). As a result, only an estimated 1,500 of the 84,000 chemicals available for commercial use have been tested so far. Moreover, the relevance of animal models to human carcinogenicity risk has been seriously questioned (Raghava et al. 2014).

### Novel Approaches for Carcinogenicity Testing

The short-term tests currently used to predict a chemical's ability to induce cancer are implemented based on scientific evidence that emerged in the 1970s, when links between DNA damage and mutation were described. Accordingly, these screening methodologies firstly aimed at identifying genotoxic agents under the premise that

such agents would most likely pose cancer risks in humans (Guyton et al. 2009). Novel approaches aim at identifying all types of carcinogens, but with varying efficacy.

#### Structure-activity relationship (SAR)

As for genotoxicity, *in silico* and structure alert methods have been proposed, with the same advantages and limitations. The OECD principles for the validation of such models must be defined by a precise endpoint, an unambiguous algorithm, goodness-of-fit, robustness, predictivity, and applicability domain (Hernandez et al. 2009; Ouedraogo et al. 2012).

#### Replicative DNA synthesis (RDS)

In eukaryotic cells, the regulatory mechanisms for DNA replication are crucial to control the cell cycle (Rizwani and Chellappan 2009); as numerous (non-genotoxic) carcinogens are mitogenic inducers, an increase in cellular proliferation can be investigated by measuring the rate of replicative DNA synthesis upon exposure of cell cultures to tested agents. The major advantages of the RDS test are the *in vivo* response to the agent and the short duration of the assay. On the other hand, disadvantages are characterized by the requirement for high doses, false positives obtained because of regenerative cell proliferation (due to acute toxicity), and false negatives obtained if the studied organ is not the primary target for the agent. For these reasons, the RDS test should be performed in conjunction with other short-term assays (Hernandez et al. 2009).

#### *In vitro* cell transformation assay

These assays detect the carcinogenic potential of a chemical through morphological transformation of primary cultured cells. The main advantages of this assay are the use of a normal cell line, the low spontaneous transformation, the capacity for metabolic activation, the rapidity of phenotypic changes, and reproducibility. On the other hand, the major disadvantage is that the induction of cells' transformation is not yet fully understood. Thus, results of this assay need to be evaluated with caution. Other disadvantages include inter- and intra-laboratory variations due to subjectivity in the scoring of transformed cells, the requirement for regular preparation of primary cultures, variation in cloning efficiency, and transformation frequency due to the composition of culture serums and the use of an initiation-promotion protocol in order to enhance the transformation frequency (Hernandez et al. 2009; OECD 2014).

#### Toxicogenomics

There is evidence that suggests that gene expression profiles in model organisms or cells exposed to various compounds reflect underlying biological mechanisms of action and can be used in higher throughput assays to predict toxicity and, notably, carcinogenicity. Predicting the carcinogenicity of genotoxic and non-genotoxic compounds has been assessed from the expression profiles of exposed cell cultures, tissues, and animals, indicating that gene expression-based carcinogenicity prediction is possible (Hernandez et al. 2009; Raghava et al. 2014). Toxicogenomic methods have progressed to the extent that it may be possible to use them in acute or sub-chronic studies to predict carcinogenicity. Several

research groups have recently identified cancer-relevant gene sets that can discriminate carcinogenic from non-carcinogenic compounds. Proof-of-concept studies using advances in toxicogenomic have provided an initial demonstration of the utility of these assays as predictive tools. However, further exploratory research, as well as validation efforts, are still needed (Guyton et al. 2009).

## Genotoxic and Carcinogenic Herbal Products

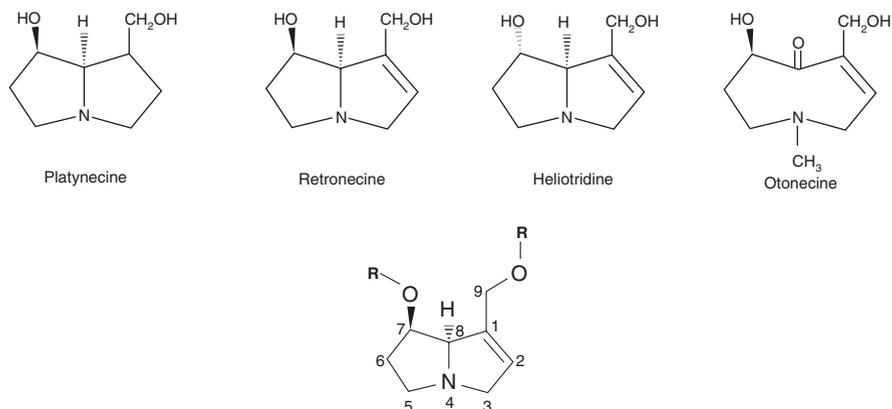
Although genotoxicity data are being generated for a growing number of medicinal plants, relatively few herbs have been proven to be carcinogenic. Until now, carcinogenicity information is still lacking, and safety information mostly relies on genotoxicity testing; some carcinogens (notably indirect) are probably not detected, which presents a rather unsatisfying situation.

### *Pyrrrolizidine Alkaloids*

Pyrrrolizidine alkaloids (PA), secondary metabolites found in 12 Angiosperm plant families, are produced for defense against herbivore insects. To date, more than 660 pyrrrolizidine alkaloids and their N-oxide derivatives have been identified in over 6,000 plants grown virtually worldwide, including Africa, the West Indies, China, Jamaica, Canada, Europe, New Zealand, Australia, and the U.S (Xia et al. 2008; Wang et al. 2005a, b). The majority of these compounds are found in different genera from three botanical families: Boraginaceae, Compositae (Asteraceae) and Leguminosae (Fabaceae) (Wang et al. 2005a; Fu et al. 2002). The genus *Senecio* (Compositae) is particularly concerned. PAs-containing plants are probably the most common poisonous plants affecting livestock, wildlife, and humans, and PAs are among the first naturally occurring carcinogens identified in plants (Xia et al. 2008). People are exposed to PAs not only by the consumption of traditional medicines or herbal teas made from PA-containing plants, such as comfrey (*Symphytum officinale* L.) (Xia et al. 2008), but also by the consumption of contaminated human foodstuffs such as milk, honey, grains, herbal medicines, and dietary supplements (Wang et al. 2005b).

The classification of PAs is mostly based on the identity of the necine base, the presence or absence of a macrocyclic structure esterifying the alcohol groups, and the number of its members (e.g., 11-, 12- or 13-membered macrocycles), stereochemistry, and patterns of hydroxylation (Langel et al. 2010) (Fig. 9.2).

Pyrrrolizidine alkaloids, particularly those from plants such as *Senecio*, *Crotalaria*, *Heliotropium* and *Amsinckia*, are highly toxic compounds, exhibiting acute toxicity, chronic toxicity and genotoxicity. Acute toxicity results in hepatic veno-occlusive disease, causing massive hepatotoxicity with hemorrhagic necrosis. Chronic poisoning takes place mainly in the liver, lungs, and blood vessels, and in some instances the kidneys, pancreas, gastrointestinal tract, bone marrow, and brain. Exposure over a longer period of time causes cell enlargement (megalocytosis),



**Fig. 9.2** Common necine bases of pyrrolizidine alkaloids (Fu et al. 2002)

veno-occlusion in liver and lungs, steatosis, nuclei enlargement with increasing nuclear chromatin, loss of metabolic function, inhibition of mitosis, proliferation of biliary tract epithelium, liver cirrhosis, nodular hyperplasia, and adenomas or carcinomas (Wang et al. 2005b; Langel et al. 2010; Mädge et al. 2015). PAs require metabolic activation to exert their genotoxicity and tumorigenicity. Upon ingestion, 1,2-unsaturated PAs (Fig. 9.2: Common necine bases of pyrrolizidine alkaloids) (Wang et al. 2005b) are oxidized by cytochromes P-450 to reactive pyrrolic bifunctional electrophiles that are potent DNA linkers, an event reputed critical in their toxicity and carcinogenesis. The platynecine-type pyrrolizidine alkaloids that do not harbor a 1–2 double bond have been found to be non-genotoxic. *In vivo* and/or *in vitro* metabolism of the tumorigenic retronecine-type (e.g., riddelliine, retrorsine, and monocrotaline), heliotridine-type (e.g., lasiocarpine) and otonecine-type (e.g., clivorine) pyrrolizidine alkaloids all generate a common set of 6,7-dihydro-7-hydroxy-1-hydroxymethyl-5H-pyrrolizine (so-called “DHP”)–derived DNA adducts responsible for tumor induction (Xia et al. 2008), and for most of the genotoxicity of the parent pyrrolizidine alkaloids (Fu et al. 2002). There are three principal metabolic pathways, mainly in the liver (CYP3A and CPY2B6 isozymes) (Fu et al. 2002): (1) hydrolysis of the ester functional group to form the necine bases; (2) oxidation of the necine bases to the corresponding necine N-oxides (heliotridine type and retronecine type); and (3) hydroxylation at the C-3 or C-8 position of the necine bases to form 3- or 8-hydroxynecine derivatives followed by dehydration, to form the corresponding dehydropyrrolizidine (pyrrolic) derivatives. The third pathway is generally considered to be the metabolic activation responsible for intoxication, whereas N-oxidation and hydrolysis are considered to be detoxifying pathways; pyrrolic ester metabolites are very reactive and can bind to one or two molecules of glutathione to form glutathione conjugates for excretion, which is a further detoxification pathway (Fu et al. 2002). The genotoxicity of pyrrolizidine alkaloids includes DNA binding, DNA cross-linking, DNA-protein cross-linking, mutagenicity, and carcinogenicity (Mädge et al. 2015).

## *Volatile Alkenylbenzenes from Essential Oils*

### **Safrole**

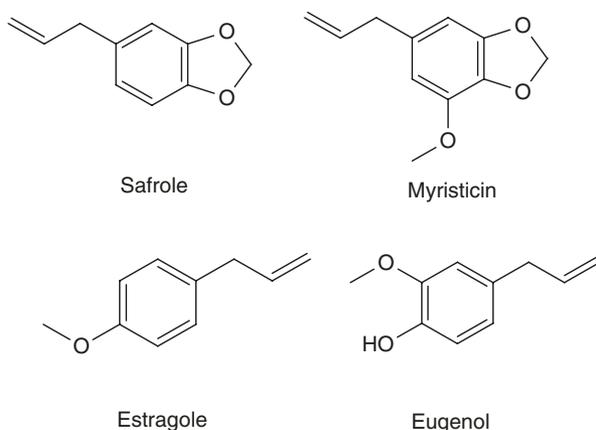
Safrole (Fig. 9.3) is a phenylpropenic compound constituting up to 80% of the essential oil of sassafras (*Sassafras albidum* (Nutt.) Nees) root bark, a tree native to the northeast U.S. that is used for medicinal and culinary purposes, especially as a flavoring agent for beverages such as root beer (Segelman et al. 1976). It is found in other species, such as nutmeg and mace (*Myristica fragrans* Houtt.), *Ocotea pretiosa* Mez. (synonym of *O. odorifera* (Vell.) Rohwer), *O. cymbarum* Kunth, *Cinnamomum camphora* (L.) J. Presl (used for the production of camphor), in betel quid (leaves of *Piper betle* L.), and in areca nut (*Areca catechu* L.). Betel quid and areca nut are widely chewed in southeast Asian countries for their addictive psycho-stimulating effects; their regular consumption has, however, been linked to a 50-times increase in the prevalence of oral cancers (Thomas and MacLennan 1992; Chen et al. 1999). Safrole has also been shown to be a weak hepatocarcinogen (Homburger et al. 1965; Miller and Miller 1976; Wislocki et al. 1977). The carcinogenic effects of safrole have been recognized since 1960, when the U.S. FDA prohibited its use in food (U.S. Food and Drug Administration 1973). The IARC classifies betel quid and areca nut as acknowledged carcinogens to humans (IARC 2004).

The genotoxicity of safrole was further investigated on mammalian cells; it was found to induce chromosomal aberrations, gene mutations, and sister chromatid exchange (European Commission: Scientific Committee on Food 2002) and to trigger unscheduled DNA synthesis in cultured rat hepatocytes – but not in HeLa cells – and DNA damage (single-strand breaks) in cultured rat hepatocytes. The genotoxicity is not mediated through safrole itself, but rather from its activation into 1'-hydroxysafrole by cytochromes P450 2C9 and 2E1 (Ueng et al. 2004). This compound is subsequently sulfonated into an unstable sulfuric acid ester capable of forming adducts with DNA (Chung et al. 2008). Other oxidized metabolites, such as 1'-acetoxysafrole, safrole-2',3'-oxide, 1'-acetoxysafrole, and 1'-oxosafrole are also suspected of being genotoxic (European Commission: Scientific Committee on Food 2002).

*In vivo*, safrole is able to induce chromosome aberrations, sister chromatid exchange, and DNA adducts in the hepatocytes of rats (Daimon et al. 1998). These DNA adducts, tentatively identified as N<sup>2</sup>-(trans-isosafrol-3'-yl)2'-deoxyguanosine and N<sup>2</sup>-(safrol-1'-yl)2'-deoxyguanosine, suggest that safrole is a genotoxic carcinogen in the liver. The presence of these adducts was effectively confirmed in the hepatic tissues from patients who developed hepatocellular carcinoma (Chung et al. 2008).

In another *in vivo* study, myristicin (methoxy-safrole, the major flavoring compound of nutmeg) along with safrole, were shown to induce hepatic DNA adducts in adult and fetal mice (Randerath et al. 1993). Transplacental passage of safrole's reactive metabolites was highlighted by the presence of DNA adducts in the livers of fetal mice whose mothers were exposed to safrole (Randerath et al. 1989). It is suggested that the dosages of safrole and myristicin ingested during the consumption of nutmeg are significantly lower than those that could be associated with psychogenic and toxic effects (Bruneton 2009).

**Fig. 9.3** Molecular structures of safrole, myristicin, estragole, and eugenol



### Estragole

Estragole (Fig. 9.3) is present in many culinary herbs, including anise, star anise, basil, bay, tarragon, fennel, and marjoram. Widespread human exposure to estragole occurs through the consumption of these herbs and through the use of their essential oils as flavors and fragrances in numerous foods, cosmetics, and other consumer products (EFSA 2009). Previously recognized as safe and approved by the U.S. FDA for food use, estragole and its metabolites have been shown to be mutagenic in bacterial systems (Ames test) and to produce hepatomas in a susceptible strain of mice. The carcinogenicity of estragole proceeds through a genotoxic mechanism upon liver metabolism into 1'-hydroxyestragole and several epoxide compounds; both estragole and its hydroxylated metabolite induce hepatic tumors in CD-1 or B6C3F1 mice either after dietary chronic exposure or after i.p. or s.c. injections, prior to or after weaning (males appear to be more susceptible than females) (Council of Europe 2005). Further strong supporting evidence of carcinogenicity comes from comparison with compounds structurally similar to estragole (e.g., safrole, methyleugenol), which produce liver tumors and tumors at other sites in rodents.

### Eugenol

Eugenol (Fig.9.3) is a widely distributed component of essential oils. It is a major constituent of clove oil and is found in several spices including basil, cinnamon, and nutmeg (Zhou et al. 2013). It has been used since at least the nineteenth century, primarily as a flavoring agent in a variety of foods, pharmaceutical products, and as an analgesic and antiseptic in dental care.

Eugenol has been investigated for its carcinogenicity in mice and rats by oral administration of a diet containing various eugenol concentrations. At high dosages (diets containing 12,000 ppm of eugenol), it induced a significant increase in the incidence of liver tumors in female mice, whereas in males, the increase was significant only for those receiving the lower dosage (dietary level of 3,000 ppm of

eugenol) (Carcinogenesis Studies of Eugenol 1983). Cytochrome P450-catalyzed metabolism has been suggested as a possible major bioactivation pathway *in vitro* (Munerato et al. 2005).

Other studies in mice by oral administration, skin application and intraperitoneal injection were inadequate for an evaluation of carcinogenicity, mainly due to the short duration of treatment. Thus there is only limited evidence of the carcinogenicity of eugenol in experimental animals. In the absence of epidemiological data, no evaluation could be made on the carcinogenicity of eugenol to humans. By contrast, methyl-eugenol – a derivative of eugenol also found in numerous dietary herbs – has shown clearer evidence of its hepatocarcinogenic activity in rodents (NTP 2000), which could be tentatively explained by the formation of DNA adducts (Williams et al. 2013).

## Asarones

Asarones ( $\alpha$ -,  $\beta$ - and  $\gamma$ -asarone; Fig. 9.4) are alkenylbenzenes isolated from a wide variety of herbs, including *Acorus calamus* L., *Acorus tatarinowii* Schott, *Asarum europaeum* L., *Asarum forbesii* Maxim., *Mosannonna depressa* (Baill.) Chatrou, *Orthodon asaroniferum* Fujita, *Orthodon isomyristicineferum* Fujita<sup>1</sup> or *Piper lolot* DC (Zhou et al. 2013; Niir 2003; Chamorro et al. 1998).

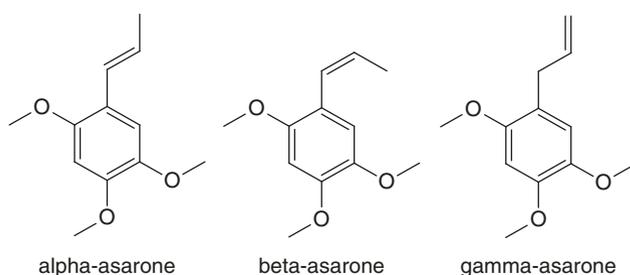
$\alpha$ -asarone is an acknowledged hypolipidemic agent (Cassani-Galindo et al. 2005) that inhibits the hepatic 3-hydroxy-3-methylglutaryl coenzyme A reductase (Rodríguez-Paez et al. 2003); proposed for the prevention of atherosclerotic disease, the compound proved, however, to be genotoxic. The carcinogenic activity of  $\beta$ -asarone has been known since a 1967 toxicity study of *Acorus calamus* L. root oil in rodents. The herb contains up to 80% of  $\beta$ -asarone and was formerly used as a flavoring agent in food and beverages (Taylor et al. 1967; Abel and Göggelmann 1986).

Numerous *in vitro* works also confirmed the genotoxicity of  $\alpha$ -asarone. Exposure of murine connective tissue cells (L929 cell line) induced DNA fragmentation measured by the comet assay; also, in human lymphocytes, increased sister chromatid exchanges were observed (Morales-Ramírez et al. 1992). The Ames test does not denote any mutagenic effect of  $\alpha$ -asarone (Marczewska et al. 2013) unless the compound is metabolically activated by preincubation with the S9 fraction (Cassani-Galindo et al. 2005), through cytochrome P450-mediated hydroxylation and sulfation. The subsequent loss of the sulfate moiety generates carbonium cations, which are able to react with DNA, thus triggering a genotoxic potential. Similarly, chromosomal aberrations were observed in human lymphocytes when  $\beta$ -asarone was metabolically activated (Abel and Göggelmann 1986). Due to its moderate capacity of increasing sister chromatid exchange,  $\alpha$ -asarone has been shown to be mutagenic in both human lymphocytes *in vitro* and murine bone marrow cells *in vivo* (Kevekordes et al. 2001). The  $\alpha$ -asarone reaches the gonads of male rats,

---

<sup>1</sup>These are not accepted botanical names; Fujita has classified *Orthodon* species according to their essential oils composition and, to the best of our knowledge, no relationship between botanical identification and essential oil classification has been established (Niir 2003).

**Fig. 9.4** Molecular structures of  $\alpha$ -,  $\beta$ - and  $\gamma$ -asarones



affects the concentration and motility of spermatozoids and induces a teratogenic activity (Unger and Melzig 2012); the pregnancies developing after mating with exposed male rats resulted in an increased incidence of post-implantation loss and fetal malformations (Abel and Göggelmann 1986; Unger and Melzig 2012). An induction of micronuclei formation was observed in human hepatoma cells (Hep G2) and increased with exposure to  $\alpha$ -asarone (Kevekorde et al. 2001). This result is, however, subject to controversy: using the same cell line, another team found that  $\beta$ -asarone – but not  $\alpha$ -asarone – induced the formation of micronuclei (Unger and Melzig 2012). Furthermore,  $\alpha$ - and  $\beta$ -asarones showed different cytotoxic profiles as revealed by a cellular proliferation (BrdU) assay; the more pronounced cytotoxicity of  $\alpha$ -asarone was tentatively explained by an increased metabolism into the cytotoxic but non-genotoxic 2,4,5-trimethoxycinnamic acid (Hasheminejad and Caldwell 1994).

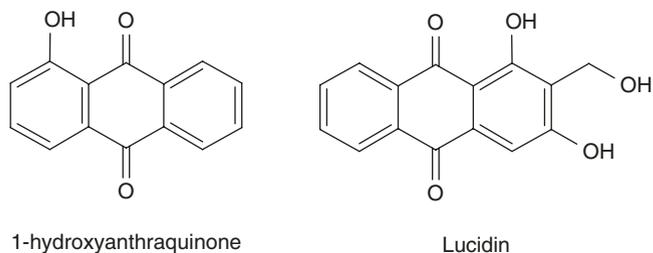
*In vivo*, asarones triggered unscheduled DNA synthesis in rat hepatocytes, suggesting genotoxicity and reinforcing the assumption of a hepatocarcinogenic potential (Hasheminejad and Caldwell 1994; Howes et al. 1990). The unscheduled DNA synthesis could be prevented when a cytochrome P450 inhibitor (cimetidine) was administered concomitantly.

### ***Anthraquinones from Rubia tinctorum L. and Morinda officinalis F.C. How***

*Rubia tinctorum* L. (madder) is a plant that grows in southern Europe, western Asia, and North Africa, and is cultivated elsewhere. Its roots, known as “madder roots”, are used for dyeing (red coloring matter from roots), treating kidney and bladder stones, as a laxative, as a mild sedative and for menstrual and urinary disorders (IARC 2002). Anthraquinones are the main bioactive compounds found in *Rubia tinctorum* L.

The fresh roots of *Morinda officinalis* F.C. How have been used as a Chinese folk medicine for their tonic and analgesic properties. A number of compounds have been isolated from *M. officinalis*, including anthraquinones, terpenoids and scopoletin (IARC 2002; Zhang et al. 2010). Among the compounds found in these two herbs, 1-hydroxyanthraquinone and 1,3-dihydroxy-2-hydroxymethylanthraquinone (lucidin) are known to be potential carcinogens (Fig. 9.5).

**Fig. 9.5** Molecular structures of two genotoxic anthraquinones



1-hydroxyanthraquinone has been isolated from the roots of *Rubia cordifolia* L., *Morinda officinalis* F.C. How and *Damnacanthus indicus* C.F.Gaertn., from the heartwood of *Tabebuia avellanadae* (Mart. ex DC.) Mattos, and the herb of *Cassia occidentalis* L. In rats, 1-hydroxyanthraquinone has also been identified as a metabolite of alizarin primeveroside, found in *Rubia tinctorum*. Lucidin has been identified in plants from several genera, such as *Rubia*, *Coprosma*, *Morinda*, *Galium*, *Hymenodictyon* and *Commitheca*.

Madder root caused an increase in hepatocellular adenomas, and adenomas and carcinomas of the renal cortex in male and female rats in a single experiment. The 1-hydroxyanthraquinone is capable of inducing DNA repair synthesis in rat hepatocytes, suggesting a genotoxic potential (Kawai et al. 1986). Following oral administration, it induced adenocarcinomas of the large intestine, highlighting a carcinogenic activity (IARC 2002). Although no data have been obtained regarding the carcinogenicity of lucidin, the compound is highly suspected to portray similar properties.

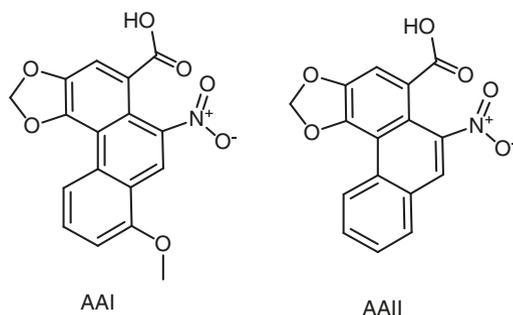
Many medicinal plants used as laxatives (senna, cascara, frangula, rhubarb, and aloe) harbor anthraquinone glycosides as active principles (Bruneton 2009). Very little is known about their potential carcinogenicity, and these plants may probably be considered to be safe. Nevertheless, 1,8-dihydroxyanthraquinone (hydrolysis product of sennosides, the laxative ingredients of senna), formerly marketed as a laxative medicine (Dantron®), was withdrawn from the market in the United States in 1987 after it was shown to cause intestinal tumors *in vivo* (National Toxicology Program 2011).

### ***Aristolochic Acids***

Aristolochic acids (AA) are phenanthrene cyclic molecules found throughout herbs belonging to the *Aristolochia* and *Asarum* genera (Fig. 9.6). They are known for their nephrotoxicity as well as their genotoxic, mutagenic, and carcinogenic potential (Bruneton 2005; Barnes et al. 2007; Michl et al. 2014; Heinrich et al. 2009), and they have therefore been listed as poisonous plants and are prohibited in many countries (Zhou et al. 2013).

Species such as *Aristolochia clematitis* L. or *Aristolochia serpentaria* L. have been traditionally used in Europe as diuretic, emmenagogue, or oxytocic herbal medicines. Despite their toxic effects – which have only been confirmed in the 1960s by *in vivo* models (Bruneton 2005) – *Aristolochia* species have been included in numerous traditional medicines worldwide and are still frequently identified as

**Fig. 9.6** Structures of AAI and AAI



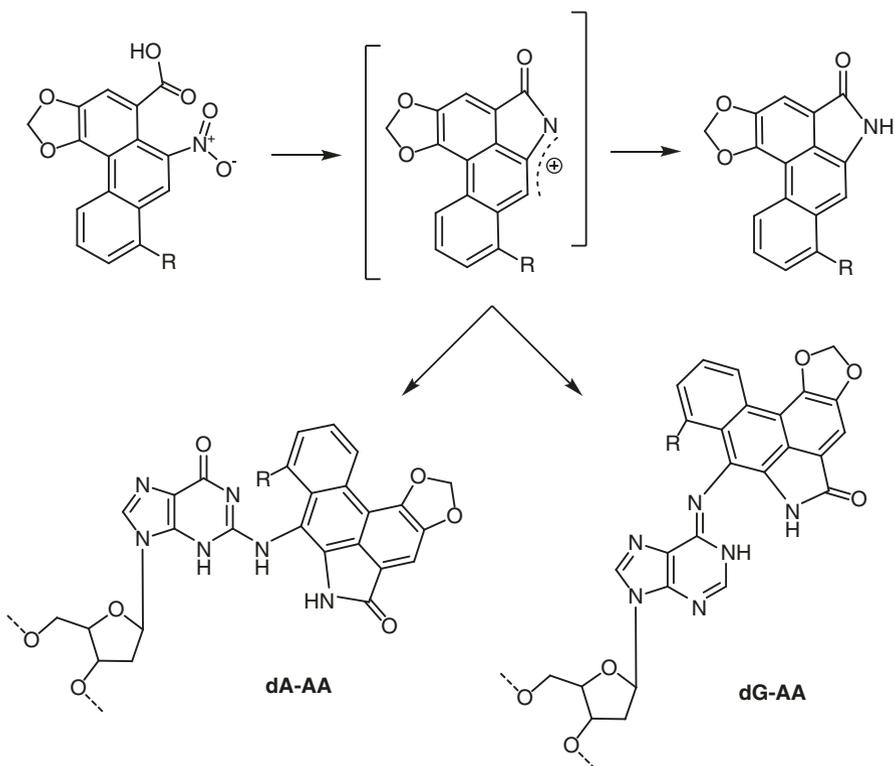
responsible for nephrotoxicity and/or carcinogenicity cases; indeed, in TCM *Aristolochia fangchi* Y.C.Wu ex L.D. Chow and S.M. Hwang has been used as a diuretic and for the treatment of rheumatism (Heinrich et al. 2009). The so-called “aristolochic acid nephropathy” (AAN) outbreak that took place in Belgium in the 1990s highlighted the nephrotoxic potential of *Aristolochia* species; a cohort of about 120 patients experienced a rapidly progressing renal interstitial fibrosis after administration of slimming capsules inadvertently containing *Aristolochia fangchi* in place of *Stephania tetrandra* S. Moore (Vanherweghem et al. 1993; Debelle et al. 2008). This regular intake of AA-containing herbs led to the complete loss of renal structure and function, requiring patients to undergo renal replacement therapy by dialysis or transplantation (Vanherweghem et al. 1993; Nortier et al. 2000).

During the follow-up of AAN patients, DNA adducts were identified in five kidney biopsies. Because of the involvement of these adducts in tumorigenesis, AAN patients appeared to be at risk of cancer development. This was confirmed in 1994, when cellular atypia was observed in three patients with AAN, throughout the urothelium of the kidneys removed during transplantation (Nortier et al. 2000; Nortier and Vanherweghem 2002). In 1997, from 39 patients who agreed to undergo prophylactic nephrectomy, 18 (46%) were positive for urothelial carcinoma, 19 had mild to moderate urothelial dysplasia, and 2 had normal urothelia. However, all renal samples were found positive for AA-DNA adducts, confirming (if still necessary) that the exposure to AA is responsible for the urothelial carcinoma onset.

Worldwide, 99 *Aristolochia* species have been identified as medicinal herbs used to treat a wide variety of ailments (Michl et al. 2013, 2014). In the Balkans, the consumption of contaminated wheat flour was identified as the cause of the so-called “Balkan endemic nephropathy”. In 2007, the presence of AA-DNA adducts in renal biopsies confirmed the involvement of *Aristolochia clematitis* L. at the onset of the disease (De Broe 2012). In Maghreb, *Aristolochia baetica* L. and *Aristolochia debilis* Sieb and Zucc are still frequently used for the treatment of cancer, digestive tract disorders, and diabetes (Bellakhdar 1999; Yamani et al. 2015). In China, between 1964 and 1999, only five cases of AAN related to the consumption of *A. fangchi* or *Aristolochia manshuriensis* were reported (Li and Wang 2004). In 2008, the number of cases rose to 116 (Debelle et al. 2008), and it is expected that the incidence of the disease is still increasing, as Chinese herbalists still probably use aristolochia-containing remedies despite their prohibition.

In cytoplasm, AA undergo an enzymatic nitroreduction, leading to the formation of aristolactames (Debelle et al. 2008). These reactive metabolites are capable of forming DNA adducts: the positive charge of N-acylnitrenium ions can be delocalized and can react with amine functions of the puric bases adenine and guanine (Fig. 9.7).

AA-DNA adducts can persist for years after ingestion of *Aristolochia* and have thus been proposed as potential biomarkers of exposure (Nortier et al. 2013). These carcinogenic properties of AA are supported by the formation of DNA adducts and by the characteristic transverse mutation A→T in the p53 tumor suppressor gene (Gokmen et al. 2013). This is especially the case of dA-AAI adduct, which is found more frequently and is considered to be highly mutagenic (Nortier et al. 2013). An overexpression of P53 protein has been highlighted in AAN-associated urothelial cancers, suggesting that the p53 gene was mutated (Debelle et al. 2008), as was confirmed by the identification of a specific AAG to TAG mutation in codon 139 (Lys-Stop) of exon 5 of p53 gene. In a rodent model, the A→T mutation was also observed in codon 61 of the H-ras oncogene and may be responsible for tumorigenesis as well (Debelle et al. 2008).



**Fig. 9.7** Metabolic activation of AA (AAI: R = OCH<sub>3</sub>/AAII: R = H) during which the nitroreduction leads to the corresponding aristolactams. The intermediate nitrenium cation can bind to DNA and form adducts, notably with adenine (dA-AA) and guanine (dG-AA) bases

## Summing up

Table 9.1 provides an overview of the genotoxicity and carcinogenicity demonstrated for compounds described in this chapter. In practice, substances giving positive genetic toxicity data are considered to be carcinogenic until proven otherwise. However, it has become clear that many non-carcinogenic natural compounds (e.g., the flavonoid quercetin) produce misleading positive results in regulatory genotoxicity assays (Ames test). Given the wide variety of modes of action for carcinogenicity, the evaluation of natural products and herbal extracts still has to be carried out case by case, based on the weight-of-evidence approach. This method assesses the weight of all epidemiological and experimental data available, taking into account their strengths and weaknesses (Hernandez et al. 2009; Walmsley and Billinton 2011; Guyton et al. 2009; Berg et al. 2011).

**Table 9.1** Overview of the genotoxicity and carcinogenicity for the various compounds described in this chapter

Group	Compound	Main botanical families	Genotoxicity	Carcinogenicity	References
Pyrrolizidine alkaloids	Retrorsine, Heliotrine, Monocrotaline	Boraginaceae Compositae (Asteraceae) Leguminosae (Fabaceae)	Yes	Yes	Wang et al. (2005a), Fu et al. (2002)
Alkenylbenzenes	Safrole	Lauraceae Myristicaceae	Yes	Yes	Segelman et al. (1976), Thomas and MacLennan (1992), Chen et al. (1999)
	Myristicin	Myristicaceae	Yes	Yes	Barnes (2007)
	Estragole	Asteraceae	Yes	Yes	Barnes (2007)
	Eugenol	Myrtaceae	No	Equivocal	Barnes (2007)
	Asarone	Acoraceae	Yes	Equivocal	Ouedraogo et al. (2012), Niir (2003), Chamorro et al. (1998)
	Anthraquinones	Rubiaceae	Probable	Probable	IARC (2002), Kawai et al. (1986)
Nitrophenanthrene Carboxylic acid	Aristolochic acids I and II	Aristolochiaceae	Yes	Yes	Debelle et al. (2008), Nortier et al. (2013)

## Conclusion

All effective drugs may produce adverse drug reactions, and herbal medicinal products are no exception (Liu et al. 2014); effectively, over the last decades, cases of poisoning due to herbal medicines have occurred in many countries (Zhou et al. 2013). The experience gained from traditional use is efficient enough to detect immediate or near-immediate relationships between administration and toxic effects, but is quite unlikely to detect medium- and long-term toxicities (Zhou et al. 2013). Notably, carcinogenicity and genotoxicity are not “obvious” adverse effects (such as gastrointestinal disorders or many autonomic nervous system modulations) but, as shown by the dramatic cases of *Aristolochia* poisoning, they are nonetheless dreadful for the patients’ health. Such an apparent lack of toxicity of an herbal medicine can lead to a false sense of safety, to chronic use, and to reliance on its properties.

“Traditional” medicine often recommends the use of combinations of HMP, an additional major challenge in safety assessment. Consequently, toxicity can be caused not only by an individual HMP drug, but also by the interaction between two or more HMP drugs (Liu et al. 2014). Moreover, in China and other countries (mainly Asian), it is frequently recommended to use conventional drugs concurrently with traditional herbal medicines. Drug-herb interactions are then also possible, in addition to eventual food-drug interactions (Liu et al. 2014). We have recently published potentiated genotoxic effects measured for the association of *Magnolia* and *Aristolochia* species. Both plants were present in the weight-reducing capsules taken by Belgian women in the 1990s, which may possibly explain the rapid onset of Chinese herb nephropathies observed in the 1990s (Nachtergaele et al. 2015).

Guidelines for genotoxicity or carcinogenicity assessment do not currently take such interactions into consideration. However, given the worldwide and constantly increasing use of herbal products, a better risk assessment certainly represents a very important point for the safety of patients.

## References

- Abel G, Göggelmann W (1986) Genotoxic activity of  $\beta$ -asarone and commercial calamus drugs. *Mutat Res/Environ Mutagen Relat Sub* 164(4):287
- Ames BN, Durston WE, Yamasaki E, Lee FD (1973) Carcinogens are mutagens: a simple test system combining liver homogenates for activation and bacteria for detection. *Proc Natl Acad Sci USA* 70(8):2281–2285
- Andrew AS, Burgess JL, Meza MM, Demidenko E, Waugh MG, Hamilton JW et al (2006) Arsenic exposure is associated with decreased DNA repair *in vitro* and in individuals exposed to drinking water arsenic. *Environ Health Perspect* 114(8):1193–1198
- Balmain A (2001) Cancer genetics: from Boveri and Mendel to microarrays. *Nat Rev Cancer* 1(1):77–82. doi:10.1038/35094086
- Barnes J, Anderson L, Phillipson D (2007) Herbal medicines. 3rd edn. Press P, editor, London
- Bellakhdar J (1999) In: Press I (ed) *La pharmacopée marocaine traditionnelle*. Ibis Press, Paris

- van den Berg SJPL, Restani P, Boersma MG, Delmulle L, Rietjens IMCM (2011) Levels of genotoxic and carcinogenic ingredients in plant food supplements and associated risk assessment. *Food Nutr Sci* 02(09):989–1010
- Bielas JH, Loeb KR, Rubin BP, True LD, Loeb LA (2006) Human cancers express a mutator phenotype. *Proc Natl Acad Sci* 103(48):18238–18242
- Billingtona N, Hastwellb P, Beerensc D, Birrellia L, Ellisb P, Maskelld S et al (2008) Interlaboratory assessment of the GreenScreen HC GADD45a-GFP genotoxicity screening assay: an enabling study for independent validation as an alternative method. *Mutat Res* 653(1–2):23–33
- Borner FU, Schutz H, Wiedemann P (2011) The fragility of omics risk and benefit perceptions. *Toxicol Lett* 201:249–257
- Botta A (2013) Relations entre génotoxicité, mutagenèse et cancérogenèse. In: l'Environnement IPMdSd (ed) Journées Nationales de Santé au Travail dans le BTP. Service Hospitalo-universitaire de Médecine et Santé au Travail, Marseille, pp 9–13
- Bruneton J (2005) *Plantes toxiques: végétaux dangereux pour l'homme et les animaux*. 3rd edn. Tec & Doc, editor. Lavoisier, Paris
- Bruneton J (2009a) *Pharmacognosie, phytochimie, plantes médicinales* 4e Ed. Lavoisier S.A.S., editor, Cachan
- Bruneton J (2009b) *Pharmacognosie: phytochimie, plantes médicinales*. 4th edn. Lavoisier, editor, Paris
- Brusick D (1980) *Principles of genetic toxicology*. Plenum Press, New York
- Burgio E, Migliore L (2015) Towards a systemic paradigm in carcinogenesis: linking epigenetics and genetics. *Mol Biol Rep* 42(4):777–790
- Butterworth BE (2006) A classification framework and practical guidance for establishing a mode of action for chemical carcinogens. *Regul Toxicol Pharmacol* 45(1):9–23, Research Support, Non-U.S. Gov't
- Carcinogenesis Studies of Eugenol (1983) (CAS No. 97-53-0) in F344/N rats and B6C3F1 mice (Feed Studies). *Natl Toxicol Program Tech Rep Ser* 223:1–159
- Cassani-Galindo M, Madrigal-Bujaidar E, Chamorro G, Díaz F, Tamariz J, Espinosa-Aguirre J (2005) *In vitro* genotoxic evaluation of three  $\alpha$ -asarone analogues. *Toxicol Vitro* 19(4):547–552
- Chamorro G, Garduño L, Martínez E, Madrigal E, Tamariz J, Salazar M (1998) Dominant lethal study of  $\alpha$ -asarone in male mice. *Toxicol Lett* 99(2):71–77
- Chan S (2003) Determination of aristolochic acids in medicinal plant and herbal product by liquid chromatography–electrospray–ion trap mass spectrometry. *Talanta* 60(4):679–685
- Charles C, Chemais M, Stevigny C, Dubois J, Nachergael A, Duez P (2012) Measurement of the influence of flavonoids on DNA repair kinetics using the comet assay. *Food Chem* 135(4):2974–2981
- Charles C, Nachtergaele A, Ouedraogo M, Belayew A, Duez P (2014) Effects of chemopreventive natural products on non-homologous end-joining DNA double-strand break repair. *Mutat Res/Genet Toxicol Environ Mutagen* 768:33–41
- Chen CL, Chi CW, Chang KW, Liu TY (1999) Safrole-like DNA adducts in oral tissue from oral cancer patients with a betel quid chewing history. *Carcinogenesis* 20(12):2331–2334
- Chen T, Mei N, Fu PP (2010) Genotoxicity of pyrrolizidine alkaloids. *J Appl Toxicol* 30(3):183–196
- Cheng K-F, Leung P-C (2012) Safety in Chinese medicine research. *Open J Saf Sci Technol* 2(01):32
- Chung Y-T, Chen C-L, Wu C-C, Chan S-A, Chi C-W, Liu T-Y (2008) Safrole-DNA adduct in hepatocellular carcinoma associated with betel quid chewing. *Toxicol Lett* 183(1–3):21–27
- Cleaver JE (2005) Cancer in *xeroderma pigmentosum* and related disorders of DNA repair. *Nat Rev Cancer* 5(7):564–573. doi:10.1038/nrc1652
- Cohnheim J (1875) Congenitales, quergestreiftes muskelsarkom der nieren. *Virchows Arch* 65(1):64–69
- Combes R (2012) *In silico* methods for toxicity prediction. In: Balls M, Combes R, Bhogal N (eds) *New technologies for genotoxicity testing*. Springer US, New York city. pp 96–116

- Council of Europe (2005) Estragole. In: Active principles (constituents of toxicological concern) contained in natural sources of flavouring. Council of Europe, Strasbourg
- Coussens LM, Werb Z (2002) Inflammation and cancer. *Nature* 420(6917):860–867. doi:[10.1038/nature01322](https://doi.org/10.1038/nature01322)
- Daimon H, Sawada S, Asakura S, Sagami F (1998) *In vivo* genotoxicity and DNA adduct levels in the liver of rats treated with safrole. *Carcinogenesis* 19(1):141–146
- Davies H, Bignell GR, Cox C, Stephens P, Edkins S, Clegg S et al (2002) Mutations of the BRAF gene in human cancer. *Nature* 417(6892):949–954. doi:[10.1038/nature00766](https://doi.org/10.1038/nature00766)
- De Broe ME (2012) Chinese herbs nephropathy and Balkan endemic nephropathy: toward a single entity, aristolochic acid nephropathy. *Kidney Int* 81(6):513–515
- Debelle FD, Vanherweghem JL, Nortier JL (2008) Aristolochic acid nephropathy: a worldwide problem. *Kidney Int* 74(2):158–169
- Dixon K, Kopras E (2004) Genetic alterations and DNA repair in human carcinogenesis. *Semin Cancer Biol* 14(6):441–448
- ECVAM (2015) European union reference laboratory for alternatives to animal testing
- EFSA (2009) Advice on the EFSA guidance document for the safety 505 assessment of botanicals and botanical preparations intended for use as food supplements, based on 506 real case studies
- Ekor M (2014) The growing use of herbal medicines: issues relating to adverse reactions and challenges in monitoring safety. *F Pharmacol [Review]* 4(177):1–10
- EMA (2008) Guideline on the assessment of genotoxicity of herbal substances/preparations. London [06/02/2014]; 4–11/2. Available from: [http://www.ema.europa.eu/docs/en\\_GB/document\\_library/Scientific\\_guideline/2009/09/WC500003569.pdf](http://www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2009/09/WC500003569.pdf)
- EMA (2008) Guideline on the assessment of genotoxicity of herbal substances/preparations. London [20/04/2015]; Available from: [http://www.ema.europa.eu/docs/en\\_GB/document\\_library/Scientific\\_guideline/2009/09/WC500003569.pdf](http://www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2009/09/WC500003569.pdf)
- European Commission: Scientific Committee on Food (2002) Opinion of the scientific committee on food on the safety of the presence of safrole (1-allyl-3,4-methylene dioxy benzene) in flavourings and other food ingredients with flavouring properties. European Commission, Brussels, Contract No.: SCF/CS/FLAV/FLAVOUR/6 ADD3
- FDA (2012) Guidance for industry S2(R1) genotoxicity testing and data interpretation for pharmaceuticals intended for human use
- Fjodorova N, Vračko M, Novič M, Roncaglioni A, Benfenati E (2010) New public QSAR model for carcinogenicity. *Chem Central J* 4(Suppl 1):S3
- Friedberg EC, Wagner R, Radman M (2002) Specialized DNA polymerases, cellular survival, and the genesis of mutations. *Science* 296(5573):1627–1630
- Fu P, Xia Q, Lin G, Chou M (2002) Genotoxic pyrrolizidine alkaloids – mechanisms leading to DNA adduct formation and tumorigenicity. *Int J Mol Sci* 3:948–964
- Gokmen MR, Cosyns JP, Arlt VM, Stiborova M, Phillips DH, Schmeiser HH et al (2013) The epidemiology, diagnosis, and management of aristolochic acid nephropathy: a narrative review. *Ann Intern Med* 158(6):469–477
- Gonzalez M, Bernad A (2012) Characteristics of adult stem cells. In: López-Larrea C, López-Vázquez A, Suárez-Álvarez B (eds) Stem cell transplantation. Springer-Verlag, New York, USA, pp 103–120
- Greene N (2002) Computer systems for the prediction of toxicity: an update. *Adv Drug Deliv Rev* 54:417–431
- Guyton KZ, Kyle AD, Aubrecht J, Cogliano VJ, Eastmond DA, Jackson M et al (2009) Improving prediction of chemical carcinogenicity by considering multiple mechanisms and applying toxicogenomic approaches. *Mutat Res/Rev Mutat Res* 681(2–3):230–240
- Hanahan D, Weinberg RA (2000) The hallmarks of cancer. *Cell* 100(1):57–70
- Hanahan D, Weinberg RA (2011) Hallmarks of cancer: the next generation. *Cell* 144(5):646–674
- Hartung T (2011) From alternative methods to a new toxicology. *Eur J Pharm Biopharm* 77:338–349

- Hasheminejad G, Caldwell J (1994) Genotoxicity of the alkenylbenzenes  $\alpha$ - and  $\beta$ -asarone, myristicin and elemicin as determined by the UDS assay in cultured rat hepatocytes. *Food Chem Toxicol* 32(3):223–231
- Heinrich M, Chan J, Wanke S, Neinhuis C, Simmonds MS (2009) Local uses of *Aristolochia* species and content of nephrotoxic aristolochic acid 1 and 2 – A global assessment based on bibliographic sources. *J Ethnopharmacol* 125(1):108–144
- Hernandez LG, van Steeg H, Luijten M, van Benthem J (2009) Mechanisms of non-genotoxic carcinogens and importance of a weight of evidence approach. *Mutat Res [Review]* 682(2–3):94–109
- Hesketh R (1997) The oncogene & tumour suppressor gene factsbook. In: Hesketh R (ed) Academic press, San Diego
- Hoet P, Godderis L (2013) Genotoxicity and mutagenicity testing: a brief overview of the main tests, pitfalls and regulatory framework. In: BEMS- Belgian Environmental Mutagen Society B (ed) Toxicology as the scientific basis for management of chemical risk. BEMS, Elewijt
- Homburger F, Bogdonoff PD, Kelley TF (1965) Influence of diet on chronic oral toxicity of Safrole and butter yellow in rats. *Proc Soc Exp Biol Med* 119(4):1106–1110
- Howes AJ, Chan VSW, Caldwell J (1990) Structure-specificity of the genotoxicity of some naturally occurring alkenylbenzenes determined by the unscheduled DNA synthesis assay in rat hepatocytes. *Food Chem Toxicol* 28(8):537–542
- Hubaux R, Becker-Santos DD, Enfield KSS, Rowbotham D, Lam S, Lam WL et al (2013) Molecular features in arsenic-induced lung tumors. *Mol Cancer* 12:20
- IARC (2002) *Rubia tinctorium*, *Morinda officinalis* and anthraquinones. IARC Monogr Eval Carcinog Risks Hum 82:129–151, Some Traditional Herbal Medicines, Some Mycotoxins, Naphthalene and Styrene
- IARC (2004) Betel-quid and areca-nut chewing and some areca-nut derived nitrosamines. IARC Monogr Eval Carcinog Risks Hum 85:1–334
- IARC (2015) International agency for research on cancer: agents classified by the IARC monographs. Available from: <http://monographs.iarc.fr/ENG/Classification/>
- ICH (2014) Guidance for industry S2B genotoxicity: a standard battery for genotoxicity testing of pharmaceuticals. [03/02/2014]. Available from: <http://www.ich.org/products/guidelines>
- ICH (2015) Guideline on the need for carcinogenicity studies of pharmaceuticals S1A S1A. Available from: <http://www.ich.org/products/guidelines>
- ICH (2015) International conference on harmonisation: testing for carcinogenicity of pharmaceuticals S1B. Available from: <http://www.ich.org/products/guidelines>
- Jacobson-Kram D (2009) Cancer risk assessment approaches at the FDA/CDER: is the era of the 2-year bioassay drawing to a close? *Toxicol Pathol* 38(1):169–170
- Kahumba JRT, Okusa PN, Bakari A, Bizumukama L, Kalonji J-B, Kiendrebeogo M, Rabemenantsoa C, El Jaziri M, Williamson EM, Duez P (2015) Traditional African medicine: from ancestral knowledge to a modern integrated future? *Science* 350(6262):S61–S63
- Kawai K, Mori H, Sugie S, Yoshimi N, Inoue T, Nakamaru T et al (1986) Genotoxicity in the hepatocyte/DNA repair test and toxicity to liver mitochondria of 1-hydroxyanthraquinone and several dihydroxyanthraquinones. *Cell Biol Toxicol* 2(4):457–467
- Kevekkordes S, Spielberger J, Burghaus C, Birkenkamp P, Zietz B, Paufler P et al (2001) Micronucleus formation in human lymphocytes and in the metabolically competent human hepatoma cell line Hep-G2: results with 15 naturally occurring substances. *Anticancer Res* 21(1A):461–469
- Knudson AG (2001) Two genetic hits (more or less) to cancer. *Nat Rev Cancer* 1(2):157–162. doi:10.1038/35101031
- Langel D, Ober D, Pelsler PB (2010) The evolution of pyrrolizidine alkaloid biosynthesis and diversity in the Senecioneae. *Phytochem Rev* 10(1):3–74
- Li X, Wang H (2004) Aristolochic acid nephropathy: what we know and what we have to do. *Nephrology (Carlton)* 9(3):109–111

- Liu X, Wang Q, Song G, Zhang G, Ye Z, Williamson EM (2014) The classification and application of toxic Chinese materia medica. *Phytother Res* 28(3):334–347
- Loeb LA (2011) Human cancers express mutator phenotypes: origin, consequences and targeting. *Nat Rev Cancer* 11(6):450–457. doi:10.1038/nrc3063
- Loeb LA, Harris CC (2008) Advances in chemical carcinogenesis: a historical review and prospective. *Cancer Res* 68(17):6863–6872
- Loeb LA, Springgate CF, Battula N (1974) Errors in DNA replication as a basis of malignant changes. *Cancer Res* 34(9):2311–2321
- Mädge I, Cramer L, Rahaus I, Jerz G, Winterhalter P, Beuerle T (2015) Pyrrolizidine alkaloids in herbal teas for infants, pregnant or lactating women. *Food Chem* 187:491–498
- Magdolenova Z, Collins A, Kumar A, Dhawan A, Stone V, Dusinska M (2014) Mechanisms of genotoxicity. A review of *in vitro* and *in vivo* studies with engineered nanoparticles. *Nanotoxicology* 8(3):233–278
- Marczewska J, Drozd E, Anuszevska E, Chilmonczyk Z, Łozowicka B (2013) Assessment of the genotoxic activity of alpha-asarone and its derivatives in the comet assay. *Acta Pol Pharm* 70(2):349–354
- Martinez-Arribas F, Alvarez T, Del Val G, Martin-Garabato E, Nunez-Villar M-J, Lucas R et al (2007) Bcl-2 expression in breast cancer: a comparative study at the mRNA and protein level. *Anticancer Res* 27(1A):219–222
- Marx J (2002) Debate surges over the origins of genomic defects in cancer. *Science* 29(5581):544–546
- Maurici D, Aardema M, Corvi R, Kleber M, Krul C, Laurent C et al (2004) Timetables for the phasing-out of animal testing in the framework of the 7th amendment to the cosmetics directive: genotoxicity and mutagenicity. Commission of the European Communities, Brussels
- Mei N, Arlt VM, Phillips DH, Heflich RH, Chen T (2006) DNA adduct formation and mutation induction by aristolochic acid in rat kidney and liver. *Mutat Res/Fundam Mol Mech Mutagen* 602(1–2):83–91
- Michl J, Jennings HM, Kite GC, Ingrouille MJ, Simmonds MSJ, Heinrich M (2013) Is aristolochic acid nephropathy a widespread problem in developing countries? A case study of *Aristolochia indica* L. in Bangladesh using an ethnobotanical–phytochemical approach. *J Ethnopharmacol* 149(1):235–244
- Michl J, Ingrouille MJ, Simmonds MS, Heinrich M (2014) Naturally occurring aristolochic acid analogues and their toxicities. *Nat Prod Rep* 31(5):676–693
- Migheli F, Migliore L (2014) Epigenetic perturbations in the context of the multi-hit hypothesis of carcinogenesis. In: Maulik N, Karagiannis T (eds) *Molecular mechanisms and physiology of disease*. Springer, New York, pp 383–399
- Miller JA, Miller EC (1976) Carcinogens occurring naturally in foods. *Fed Proc* 35(6):1316–1321
- Monier R (2008) News in carcinogenesis. *C R Biol* 331(2):101–103
- Morales-Ramírez P, Madrigal-Bujaidar E, Mercader-Martínez J, Cassini M, González G, Chamorro-Cevallos G et al (1992) Sister-chromatid exchange induction produced by *in vivo* and *in vitro* exposure to alpha-asarone. *Mutat Res/Genet Toxicol Environ Mutagen* 279(4):269–273
- Muller L, Kikuchi Y, Probst G, Schechtman L, Shimada H, Sofuni T et al (1999) ICH-Harmonised guidances on genotoxicity testing of pharmaceuticals: evolution, reasoning and impact. *Mutat Res* 436:195–225
- Muller J, Decordier I, Hoet PH, Lombaert N, Thomassen L, Huaux F et al (2008) Clastogenic and aneugenic effects of multi-wall carbon nanotubes in epithelial cells. *Carcinogenesis* 29(2):427–433
- Mulwate SJ (2012) Trace elements and carcinogenicity: a subject in review. *3 Biotech* 3(2):85–96
- Munerato MC, Sinigaglia M, Reguly ML, de Andrade HH (2005) Genotoxic effects of eugenol, isoeugenol and safrole in the wing spot test of *Drosophila melanogaster*. *Mutat Res* 582(1–2):87–94

- Nachtergaeel A, Charles C, Spanoghe M, Gadenne M, Belayew A, Duez P (2013) Measurement of translesion synthesis by fluorescent capillary electrophoresis: 7, 8-Dihydro-8-oxodeoxyguanosine bypass modulation by natural products. *Anal Biochem* 440(1):23–31
- Nachtergaeel A, Poivre M, Belayew A, Duez P (2015) *In vitro* genotoxicity tests point to an unexpected and harmful effect of a Magnolia and Aristolochia association. *J Ethnopharmacol* 174(C):178–186
- NAP (2007) Applications of toxicogenomic technologies to predictive toxicology and risk assessment. Press NA, editor, Washington
- National Cancer Institute NIH (2015) Tumor microenvironment national institute of health; [22/04/2015]. Available from: <http://www.cancer.gov/dictionary?cdrid=561725>
- National Toxicology Program (2011) Danthron. *Rep Carcinog* 12:128–129
- Niir (2003) In: Research NIaN (ed) The complete technology book of essential oils (aromatic chemicals), 1st edn. Asia Pacific Business Press, Delhi
- Nortier JL, Vanherweghem JL (2002) Renal interstitial fibrosis and urothelial carcinoma associated with the use of a Chinese herb (*Aristolochia fangchi*). *Toxicology* 181–182:577–580
- Nortier JL, Martinez MC, Schmeiser HH, Arlt VM, Bieler CA, Petein M et al (2000) Urothelial carcinoma associated with the use of a Chinese herb (*Aristolochia fangchi*). *N Engl J Med* 342(23):1686–1692
- Nortier J, Pozdzik A, Roumeguere T, Vanherweghem J-L (2013) Néphropathie aux acides aristolochiques (“néphropathie aux herbes chinoises”). *EMC – Néphrologie* 10(2):1–14
- NTP (2000) NTP toxicology and carcinogenesis studies of methyleugenol (CAS NO. 93-15-2) in F344/N rats and B6C3F1 mice (gavage studies). *Natl Toxicol Program Tech Rep Ser* 491:1–412
- Nutraceuticals World (2012) Global herbal supplement market to reach \$107 billion by 2017 [29/04/2015]. Available from: [http://www.nutraceuticalsworld.com/contents/view\\_breaking-news/2012-03-07/global-herbal-supplement-market-to-reach-107-billion-by-2017](http://www.nutraceuticalsworld.com/contents/view_breaking-news/2012-03-07/global-herbal-supplement-market-to-reach-107-billion-by-2017)
- O’Brien CA, Pollett A, Gallinger S, Dick JE (2007) A human colon cancer cell capable of initiating tumour growth in immunodeficient mice. *Nature* 445(7123):106–110. doi:10.1038/nature05372
- OECD (2007) Detailed review paper on cell transformation assays for detection of chemical carcinogens. In: Environment O (ed) Health and Safety Publications
- OECD (2014) OECD guidelines for testing of chemicals [03/02/2014]. Available from: [http://www.oecd-ilibrary.org/fr/environment/lignes-directrices-de-l-ocde-pour-les-essais-de-produits-chimiques-section-4-effets-sur-la-sante\\_20745842](http://www.oecd-ilibrary.org/fr/environment/lignes-directrices-de-l-ocde-pour-les-essais-de-produits-chimiques-section-4-effets-sur-la-sante_20745842)
- Ouedraogo M, Baudoux T, Stévigny C, Nortier J, Colet J-M, Efferth T et al (2012) Review of current and “omics” methods for assessing the toxicity (genotoxicity, teratogenicity and nephrotoxicity) of herbal medicines and mushrooms. *J Ethnopharmacol* 140(3):492–512
- Pelkonen O, Xu Q, Fan T-P (2014) Why is research on herbal medicinal products important and how can we improve its quality? *J Tradit Complement Med* 4(1):1–7
- Quante M, Wang TC (2008) Inflammation and stem cells in gastrointestinal carcinogenesis. *Physiology (Bethesda)* 23:350–359
- Raghava GPS, Gusenleitner D, Auerbach SS, Melia T, Gómez HF, Sherr DH et al (2014) Genomic models of short-term exposure accurately predict long-term chemical carcinogenicity and identify putative mechanisms of action. *PLoS One* 9(7):e102579
- Randerath K, Liehr JG, Gladek A, Randerath E (1989) Use of the 32P-postlabelling assay to study transplacental carcinogens and transplacental carcinogenesis. *IARC Sci Publ* 96:189–205
- Randerath K, Putman KL, Randerath E (1993) Flavor constituents in cola drinks induce hepatic DNA adducts in adult and fetal mice. *Biochem Biophys Res Commun* 192(1):61–68
- Rizwani W, Chellappan S (2009) *In vitro* replication assay with Mammalian cell extracts. In: Chellappan S (ed) Chromatin protocols. Humana Press, New York city. pp 203–216
- Rodriguez-Paez L, Juárez-Sanchez M, Antúñez-Solís J, Baeza I, Wong C (2003) Alpha-asarone inhibits HMG-CoA reductase, lowers serum LDL-cholesterol levels and reduces biliary CSI in hypercholesterolemic rats. *Phytomedicine* 10(5):397–404

- Rossmann TG (2003) Mechanism of arsenic carcinogenesis: an integrated approach. *Mutat Res/Fundam Mol Mech Mutagen* 533(1):37–65
- Sarasin A (2003) An overview of the mechanisms of mutagenesis and carcinogenesis. *Mutat Res/Rev Mutat Res* 544(2–3):99–106
- Segelman AB, Segelman FP, Karliner J, Sofia RD (1976) Sassafras and herb tea. Potential health hazards. *JAMA* 236(5):477
- Shaked Y, Ciarrocchi A, Franco M, Lee CR, Man S, Cheung AM et al (2006) Therapy-induced acute recruitment of circulating endothelial progenitor cells to tumors. *Science* 313(5794):1785–1787
- Shen S, Wang C, Weinfeld M, Le XC (2013) Inhibition of nucleotide excision repair by arsenic. *Chin Sci Bull* 58(2):214–221
- Smith K (1992) Spontaneous mutagenesis: experimental, genetic and other factors. *Mutat Res* 277(2):139–162
- Taylor JM, Jones WI, Hagan EC, Gross MA, Davis DA, Cook EL (1967) Toxicity of oil of calamus (Jammu variety). *Toxicol Appl Pharmacol* 10(2):378–411
- Thomas SJ, MacLennan R (1992) Slaked lime and betel nut cancer in Papua New Guinea. *Lancet* 340(8819):577–578
- Tomasetti C, Vogelstein B (2015) Variation in cancer risk among tissues can be explained by the number of stem cell divisions. *Science* 347(6217):78–81
- U.S. Food and Drug Administration (1973) Database of select committee on GRAS substances (SCOGS) reviews: nutmeg and mace. FDA, Silver Spring
- Ueng YF, Hsieh CH, Don MJ, Chi CW, Ho LK (2004) Identification of the main human cytochrome P450 enzymes involved in safrole 1'-hydroxylation. *Chem Res Toxicol* 17(8):1151–1156
- Unger P, Melzig MF (2012) Comparative study of the cytotoxicity and genotoxicity of alpha- and beta-asarone. *Sci Pharm* 80(3):663–668
- Valerio LG Jr (2009) *In-silico* toxicology for the pharmaceutical sciences. *Toxicol Appl Pharmacol* 241:356–370
- Vanherweghem JL, Tielemans C, Abramowicz D, Depierreux M, Vanhaelen-Fastre R, Vanhaelen M et al (1993) Rapidly progressive interstitial renal fibrosis in young women: association with slimming regimen including Chinese herbs. *Lancet* 341(8842):387–391
- Vineis P, Schatzkin A, Potter JD (2010) Models of carcinogenesis: an overview. *Carcinogenesis* 31(10):1703–1709
- Virchow R (1855) Editorial Archive fuer pathologische. *Anatomie und Physiologie fuer klinische Medizin* 8:23–54
- Walmsley RM, Billinton N (2011) How accurate is in vitro prediction of carcinogenicity? *Br J Pharmacol* 162(6):1250–1258
- Wang T-L, Rago C, Silliman N, Ptak J, Markowitz S, Willson JKV et al (2002) Prevalence of somatic alterations in the colorectal cancer cell genome. *Proc Natl Acad Sci* 99(5):3076–3080
- Wang YP, Yan J, Beger RD, Fu PP, Chou MW (2005a) Metabolic activation of the tumorigenic pyrrolizidine alkaloid, monocrotaline, leading to DNA adduct formation in vivo. *Cancer Lett* 226(1):27–35 [Research Support, Non-U.S. Gov't Research Support, U.S. Gov't, Non-P.H.S.]
- Wang Y-P, Yan J, Fu PP, Chou MW (2005b) Human liver microsomal reduction of pyrrolizidine alkaloid N-oxides to form the corresponding carcinogenic parent alkaloid. *Toxicol Lett* 155(3):411–420
- Williams GM, Iatropoulos MJ, Jeffrey AM, Duan JD (2013) Methyleugenol hepatocellular cancer initiating effects in rat liver. *Food Chem Toxicol* 53:187–196
- Wislocki PG, Miller EC, Miller JA, McCoy EC, Rosenkranz HS (1977) Carcinogenic and mutagenic activities of safrole, 1'-hydroxysafrole, and some known or possible metabolites. *Cancer Res* 37(6):1883–1891
- Wu J, Starr S (2014) Low-fidelity compensatory backup alternative DNA repair pathways may unify current carcinogenesis theories. *Future Oncol* 10(7):1239–1253

- Wu S, Powers S, Zhu W, Hannun YA (2016) Substantial contribution of extrinsic risk factors to cancer development. *Nature* 529(7584):43–47
- Xia Q, Yan J, Chou MW, Fu PP (2008) Formation of DHP-derived DNA adducts from metabolic activation of the prototype heliotridine-type pyrrolizidine alkaloid, heliotrine. *Toxicol Lett* 178(2):77–82, Research Support, U.S. Gov't, Non-P.H.S
- Yamani A, Bunel V, Antoine M-H, Husson C, Stévigny C, Duez P et al (2015) Substitution between *Aristolochia* and *Bryonia* genus in North-Eastern Morocco: toxicological implications. *J Ethnopharmacol* 166:250–260
- Zhang HL, Zhang QW, Zhang XQ, Ye WC, Wang YT (2010) Chemical constituents from the roots of *Morinda officinalis*. *Chin J Nat Med* 8(10):1–4
- Zhou J, Ouedraogo M, Qu F, Duez P (2013) Potential genotoxicity of traditional Chinese medicinal plants and phytochemicals: an overview. *Phytother Res* 27(12):1745–1755