In vitro and in vivo studies of natural products: A challenge for their valuation. 
The case study of chamomile (Matricaria recutita L.)

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A B S T R A C T
Medicinal plant research is universally on the rise. Researchers, as well as the general public, recognize that natural products, predominantly those derived from plants, may exhibit health benefits. The tendency is to consider natural products as non-toxic and presenting fewer side effects than those used by conventional medicine. However, information concerning the real human health benefits of natural products is yet seldom available, which is a drawback for their possible valuation. Chamomile is one of the most widely used medicinal plants and its sesquiterpenic-related products are an example of this informative weakness. Several health benefits have been claimed for chamomile extracts and for a large number of sesquiterpenic compounds known to occur in chamomile. However, a deep knowledge concerning the compounds responsible for each specific effect, as well as the mechanisms behind them has not been stated, or, if it exists, is dispersed in literature. Thus, this review comprises a deep survey on the reported potential health benefits of chamomile-related sesquiterpenic compounds, and takes into account the models used for their evaluation: in vitro or in vivo. In spite of the relevance of the in vitro and animal studies reported in literature, where the data obtained are very promising concerning the potential health benefits of chamomile-related sesquiterpenic compounds, their extension to human trials is essential. Several aspects related to this actual challenge are discussed.

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1. Introduction

Focus on medicinal plant research has been increasing all over the world. People are turning to natural products; predominantly those derived from plants for their health care, due to the growing recognition that these natural products are mainly non-toxic, have lesser side effects than synthetic drugs, and are accessible at affordable prices (Milner, 1999; Chandrasekaran et al., 2010). In traditional medicine, plant formulations and combined extracts of plants are used for the treatment of a wide variety of diseases (Ansarullah et al., 2009). This therapeutic approach, although considered to be an alternative to conventional medicine, is still ignored by medical practice due to the lack of suitable scientific and clinical evidence (Fontanarosa and Lundberg, 1998; Yuan and Lin, 2000). In order to assess the efficacy and safety of products, numerous in vitro studies need to be carried out, which although very useful are not enough. The use of animal and human models is essential to scientifically prove the efficacy and safety of natural products on humans (Schneeman, 2007). The use of animals or humans to determine the toxicity of a pharmaceutical/biopharmaceutical or medical device is a required and determinant part for the validation and safe use of the natural products on humans (Alabaster, 2002).

The definition of in vitro and in vivo studies depends on the experimental model used. The in vivo studies refer to the characterization and analysis of molecules and biological systems in the context of intact organisms such as animals and humans. On the other hand, in vitro studies refer to the manipulation of organs, tissues, cells, and biomolecules under controlled artificial conditions (Lodish et al., 2004). In vivo studies are often employed for observing the overall effects of an experiment on a living organism (Novakovski, 2004). Unfortunately, in vivo studies are far more expensive, and often more difficult to control, than those performed in vitro. Despite the highlighted drawbacks of the in vitro model systems, they are more suitable than the in vivo models for the understanding of the mechanisms of drug-induced toxicity due to their lower structural and functional heterogeneity (Davila et al., 1998).

Plants have been used throughout history as the primary source of food, fuel, and medicine, among other purposes. Today, plants, plant extracts, and plant-derived components are being used in a multitude of herbal medicines and phytopharmaceuticals (Van der Kooy et al., 2009). Taking into consideration the Directive 2002/46/EC (Directive, 2002) on Food Supplements, and Directive 2004/24/EC (Directive, 2004) on herbal medicinal products, a medicinal product has to be identified by carefully interpreting the data on physiological versus pharmacological, and health versus disease conditions on dose/concentration bases.

For many of the medicinal plants of current interest, a primary focus of research to date has been in the areas of horticulture, phytochemistry, and pharmacognosy. Horticultural research on medicinal plants has focused on developing the capacity for optimal growth in cultivation. This has been specially pertinent as many medicinal plants are still harvested in the wild and, in several cases, conditions for growth in controlled cultivation have not been optimized with the purpose of increasing the yields of bioactive compounds. Wild harvesting of medicinal plants can be problematic in terms of loss of biodiversity, potential variation in medicinal plant quality and, occasionally, improper plant identification with potentially severe consequences. From the perspective of plant physiology, extensive opportunities exist for basic research on medicinal plants and for the study of their phytochemical chemical production (Briskin, 2000). In the area of phytochemistry, medicinal plants have been characterized for their possible bioactive compounds, which have been isolated and subjected to detailed structural characterization. Research on the pharmacognosy of medicinal plants has also involved assays of bio-activity, identification of potential modes of action, and target sites for active phytomedicinal compounds. Matricaria recutita L., known as chamomile, is one of the most widely used medicinal plants and is included in the pharmacopeias of 26 countries all over the world (Shikov et al., 2008; Mohammad et al., 2010). This plant was used as a case study to discuss the importance that the development of in vivo studies has in the future valuation of natural products.

2. Biological activity of M. recutita L. and its sesquiterpenic compounds

M. recutita L. is an herbaceous plant that is indigenous to Europe and Western Asia (Mckay and Blumberg, 2006). Nowadays, it is mainly cultivated in Europe, South America and, to a lesser extent, in Africa (Povh et al., 2001). Several products obtained from chamomile are commercially available, such as soaps, detergents, fragrances, lotions, ointments, hair products, baked goods, confectionery, alcoholic beverages, and infusions (Mckay and Blumberg, 2006). The consumption of chamomile as infusion is rated as more than one million cups per day (Maschi et al., 2008).

A substantial part of the pharmacological effects is determined by the biologically active chemical constituents that have been identified, namely the sesquiterpenoids and the phenolic compounds (Mann and Siaba, 1986; Mckay and Blumberg, 2006). Sesquiterpenic compounds such as α-bisabolol, bisabolol oxides A and B and chamazulene and farnesene, and phenolic compounds namely the flavonoids apigenin, quercetin, patuletin, and luteolin, and their glucosides, and also coumarins (heminarin and umbelliferone), are considered to be the major bioactive compounds of chamomile (Habersang et al., 1979; Miller et al., 1996; Salamon, 2007; Lemberkovics et al., 1998; Baser et al., 2006). Among the flavonoids, apigenin is the most promising compound. It is present in very small quantities as free apigenin, but predominantly exists in the form of various glycosides (Avallone et al., 2000; Srivastava and Gupta, 2007; Srivastava et al., 2010).

The phenolic compounds of chamomile, namely the flavonoids, have been extensively studied and revised (Cushnie and Lamb, 2005; Patel et al., 2007). However, the information relating to the biological activities of sesquiterpenic compounds is still dispersed. Furthermore, chamomile inflorescences have been reported as a potential source of sesquiterpenic compounds (Orav et al., 2001; Szoke et al., 2004a,b; Rubio et al., 2006; Petronilho et al., 2011).

Sesquiterpenic compounds are extensively found in the essential oils of several plants and fruits, providing a wide spectrum of aromas, mostly perceived as very pleasant, and responsible for the aroma perception of several natural products. Sesquiterpenic compounds have also been related with medicinal plants and fruits with different health applications (Rocha et al., 2006).

Several biological effects have been attributed to M. recutita L., such as anti-microbial, antioxidant, anti-malarial, anti-mutagenic, anti-platelet, anti-chemotactic, anti-cancer, anti-inflammatory, anti-genotoxic, anti-spasmolytic, vulnerary, mildly sedative, hypcholesterolemic, beneficial for gastrointestinal, hepatic, central nervous system and autonomic nervous system, hemodynamic, and topical properties. It has been used for flatulent nervous dyspepsia, travel sickness, nasal catarrh, restlessness, and specifically for gastro-intestinal disorders associated with nervous irritability in children. It has also been applied topically for haemorrhoids, mastitis, leg ulcer treatments, renal colic, nausea, skin eruption, constipation, as a sedative, for the expulsion of parasitic worms, stomach complaints, and skin diseases (Forster et al., 1980; Salamon and Honcarov, 1994; Kintziros and Michaelakis, 1999; Di Stasi et al., 2002; Franke and Schilcher, 2005; Crotteau et al., 2006; Kroll and Cordes, 2006; Gardiner, 2007; Srivastava et al., 2009; Bhaskaran
et al., 2010; Hernández-Ceruelos et al., 2010; Rahimia et al., 2011). Chamomile extracts also revealed antioxidant activity (Hernández-Ceruelos et al., 2002; Kolekar et al., 2008). The essential oil of chamomile that contains several sesquiterpenic compounds such as α-bisabolol also revealed anti-inflammatory, anti-bacterial,antimycotic, and ulcer-protective properties (Isaac, 1979; Szelenyi et al., 1979; Yarosh et al., 2006).

These health benefits of *M. recutita* L. are associated with several groups of active components. In this review, although special attention is devoted to the chamomile-related sesquiterpenic compounds, it was considered useful to report the biological activity of *M. recutita* L. extracts, which comprise other components besides the sesquiterpenic compounds, as well as the effects reported in literature using chemical standards that correspond to those detected in chamomile. Studies that described biological effects of chemical standards like chamazulene, germacrene D, nerolidol, α-bisabolol, farnesol, bisabolol oxide A, chamaviolin and chamazulene carboxylic acid (Fig. 1) were considered (Rekka et al., 1996; Brehm-Stecher and Johnson, 2003; van Zyl et al., 2006; Simões et al., 2008; Ogata et al., 2010). For simplicity, the criterion used for systematizing bibliography was based on the experimental model used. Thus, biological activities of *M. recutita* L. are described in terms of *in vitro* and *in vivo* studies; the latter including animal models, and human trials. An overview of the existing biological activities of *M. recutita* L. is shown in Table 1.

### 2.1. *In vitro* studies

#### 2.1.1. Anti-microbial activity

Several *in vitro* studies showed the anti-microbial activity of the essential oil, the aqueous and ethanolic extracts of chamomile, and also the chemical standards known to occur in chamomile, such as nerolidol, farnesol, α-bisabolol, germacrene D, and chamazulene. Some studies showed that the essential oil extracted from chamomile exhibits anti-microbial activity against some species of bacteria, fungi, and virus. The essential oil is effective against 25 different Gram-positive and Gram-negative bacteria, including 20 strains of *Listeria monocytogenes* (Lis-Balchin et al., 1998; Mckay and Blumberg, 2006). The anti-fungal activities of the essential oil (0.5–3 g l⁻¹) are also effective against *Aspergillus* and *Fusarium* species, where the highest concentration exhibited the highest inhibition against these microorganisms (91–95% inhibition) (Soliman and Badea, 2002; Mckay and Blumberg, 2006). It has also been reported that chamomile essential oil is active...
Potential biological activities of *Matricaria recutita* L. and its sesquiterpenic compounds.

<table>
<thead>
<tr>
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<th>Type of sample</th>
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<tr>
<td>Anti-microbial activity</td>
<td>Essential oil</td>
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<td></td>
<td></td>
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2. In vivo studies
2.1. Animal model studies
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<td>Essential oil (α-bisabolol, bisabolol oxides A and B) Chemical standard</td>
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against *Helicobacter pylori* (Stamatis et al., 2003). Urease production by *H. pylori* is inhibited by *M. recutita* L. essential oil (Shikov et al., 2008). The minimal inhibitory concentration required to inhibit the growth of 90% of microorganisms (MIC<sub>90</sub>) and the minimal inhibitory concentration required to inhibit the growth of 50% of microorganisms (MIC<sub>50</sub>) were 125 g of essential oil L<sup>-1</sup> and 62.5 g L<sup>-1</sup>, respectively. *H. pylori* is able to resist the low pH of the stomach by producing urease which hydrolyses urea converting it into ammonia and carbon dioxide. Thus, an increase in the pH disturbs the mechanisms that lead to the survival of the bacteria within the stomach. The application of the oil extract influences the morphological and fermentative properties of *H. pylori* and also inhibits the colony activity of this microorganism (Shikov et al., 2008). Considering the high incidence of *H. pylori* infection around the world, mainly in less developed countries, the evaluation of the potential use of these compounds seems to be interesting, thus the extension of this test to *in vivo* studies is essential. This step should evaluate the concentrations of chamomile active components that guarantee the biological effect and prevent toxic effects. The data related to the anti-microbial activity of chamomile essential oil show that the necessary quantities of essential oil to inhibit the fungi *Aspergillus* and *Fusarium* activity (91% of inhibition – 3 g L<sup>-1</sup>) is very different from that reported for the inhibition of the bacteria *H. pylori* activity (90% of inhibition – 125 g L<sup>-1</sup>). Suggesting that the essential oil is more effective against these fungi than against this bacterium.

An ethanol extract of chamomile is able to inhibit the growth of both herpes and polio virus. In general, aqueous extracts of chamomile are more effective against moulds and yeasts, while ethanolic extracts show higher activities against bacteria (Mckay and Blumberg, 2006). In a study reported by Franke and Schöller (2005), a chamomile alcoholic extract (42 vol%, v/v) also presents antibacterial activity against Gram-positive bacteria strains such as *Staphylococcus aureus* ATCC 12600, *Streptococcus mutans*, group B *Streptococcus*, and *Streptococcus salivarius*, and Gram-negative bacteria strains such as *Klebsiella pneumoniae*, *Escherichia coli*, *Bacillus megaterium* and *Leptospira icterohaemorrhagiae*.

Several studies have been reported also using individual compounds detected in chamomile. Nerololiod, farnesol, α-bisabolol, germacrene D, and chamazulene have been investigated for their anti-microbial activity. Nerolidol, farnesol, and α-bisabolol promote the disruption of the bacterial cell membrane, allowing the permeation into the cell of exogenous solutes such as ethidium bromide (a membrane-impermeant model drug) and antibiotics (Brehm-Stecher and Johnson, 2003). The capability of these sesquiterpenoids to increase bacterial susceptibility to a number of clinically important antibiotics was also investigated. Treatment with concentrations of 0.11–0.44 g L<sup>-1</sup> of these sesquiterpenoids enhances the susceptibility of *S. aureus* to ciprofloxacin, clindamycin, erythromycin, gentamicin, tetracycline, and vancomycin, suggesting a general role for these sesquiterpenoids as enhancers of nonspecific bacterial permeability to antibiotics and antimicrobials (Brehm-Stecher and Johnson, 2003; Kamatou and Viljoen, 2010). Furthermore, other studies carried out with α-bisabolol and nerolidol show that these compounds present by themselves anti-microbial activity against *S. aureus*, *Bacillus cereus*, E. coli, and *Candida albicans*. However, higher MIC<sub>50</sub> values were obtained than those required to potentiate the antibiotic activity, namely, for α-bisabolol MIC<sub>50</sub> ranged from 8 g L<sup>-1</sup> for *C. albicans* to 32 g L<sup>-1</sup> for all bacterial species under study, and the MIC<sub>50</sub> value of nerolidol was ca. 32 g L<sup>-1</sup> for all species under study (van Zyl et al., 2006; Kamatou and Viljoen, 2010). This result shows that α-bisabolol has higher anti-fungal activity than nerolidol and similar anti-bacterial activity. Nerolidol and germacrene D have been proposed as alternative or possible synergistic compounds for current antimicrobial chemotherapeutics, showing the practical utility of natural products, found in plants, to sensitize pathogenic bacteria by increasing their susceptibility (Simões et al., 2008). Other studies reported that α-bisabolol presented the strongest antibacterial activity when compared with its oxides. It is active against *S. aureus*, *Bacillus subtilis*, *E. coli*, *Streptococcus faecalis*, and *Pseudomonas aeruginosa* and inhibits the growth of *Bacillus pheii* that were resistant against standard anti-infectives. α-Bisabolol was also shown fungicidal activity against *C. albicans*, *Trichophyton mentagrophytes*, and *Trichophyton rubrum* at a concentration of 0.10 g L<sup>-1</sup>. Chamazulene also has fungicidal activity, but at higher concentrations (Franke and Schöller, 2005).

The determination of anti-microbial activity in *vitro* allows to predict the potential use of chamomile-related sesquiterpenic compounds in an actual and relevant issue that is the resistance to antibiotics of a continuously larger number of microorganisms, however it is imperative to evaluate the necessary quantities to make the desirable effect in humans.

### 2.1.2. Antioxidant activity

Several studies have been carried out in order to evaluate the antioxidant activity of *M. recutita* L. Essential oil, ethanolic extracts, and infusions of chamomile have been tested, and all of them exhibited antioxidant activity, although the aqueous extracts exhibited significantly higher activity than the ethanolic ones (Mckay and Blumberg, 2006). Aqueous and ethanolic extracts of chamomile have a moderate activity when compared with other medicinal plants (Lee and Shibamoto, 2002; Dragland et al., 2003; Koleckar et al., 2008). The antioxidant capacity of chamomile infusions methanolic extract was reported as 44 g trolox kg<sup>-1</sup>. This value is higher than that exhibited by curinder (8.3 g kg<sup>-1</sup>), but is lower than the values reported for peppermint (197 g kg<sup>-1</sup>) and oregano (344 g kg<sup>-1</sup>) (Dragland et al., 2003). Lee and Shibamoto (2002) evaluated the antioxidant activity of an aqueous chamomile extract (20 g L<sup>-1</sup> water) using two different assay systems. In the aldehyde/carboxylic acid assay, using butylated hydroxytoluene and α-tocopherol as standards, the highest dose (0.50 g L<sup>-1</sup>) of the chamomile extract inhibited 50% of hexanal oxidation over a 40-day period. Compared with chamomile, the inhibition activities of thyme and basil were higher (100%), lavender and cinnamon were lower (5–6%), and that of rosemary was comparable (59%). In a conjugated diene assay measuring the inhibition of hydroperoxide formation from methyl linoleate (without initiators or metal catalysts), the trend of inhibition for the same extracts was similar to that observed for the hexanal oxidation. At the highest concentration (0.20 g L<sup>-1</sup>), chamomile exhibited 31% inhibition of conjugated diene formation. Koleckar et al. (2008) carried out a study with 88 ethanolic extracts from various parts of plants from European Asteraceae, including *M. recutita* L. and Chichoraeae. These plant extracts were analysed for radical scavenging activity by means of 1,1-diphenyl-2-picrylhydrazyl radical (DPPH·) test using the sequential injection analysis (SIA) methodology. The radical scavenging activity of all tested plant extracts was evaluated according to the 50% inhibitory concentration (IC<sub>50</sub>). The IC<sub>50</sub> values ranged from 0.046 g L<sup>-1</sup> for the most active sample (leaves of *Leuzea carthamoides*) to 0.542 g L<sup>-1</sup> for the least active sample (inflorescences of *Centauraea cyanus*). The IC<sub>50</sub> value for *M. recutita* L. was 0.152 g L<sup>-1</sup>, which suggests a potential antioxidant activity.

Data on the antioxidant activity of chamazulene, nerolidol and α-bisabolol are also available. Rekka et al. (1996) showed that chamazulene inhibits Fe<sup>2+</sup>-ascorbate-induced lipid peroxidation, measured by the thiobarbituric acid reactive substances (TBARS) assay, in a concentration and time dependent mode (IC<sub>50</sub> = 3.32 mg L<sup>-1</sup> after 45 min incubation). In the same study, chamazulene was also shown to inhibit the autoxidation of dimethyl sulfoxide and to have a weak capacity to interact with the stable free radical diphenylpicrylhydrazyl (DPPH·). Similarly, nerolidol and α-bisabolol, both presenting IC<sub>50</sub> >100 mg L<sup>-1</sup>,
have also exhibited antioxidant activity (van Zyl et al., 2006), which is, however, lower than that exhibited by chamazulene. Braga et al. (2009) investigated the antioxidant activity of α-bisabolol by studying the ability of this compound to interfere with the production of reactive oxygen species (luminol-amplified chemiluminescence, LACL) during human neutrophil bursts induced by both corpusculate (C. albicans) and soluble stimulants (N-formyl-methionyl-leucyl-phenylalanine, fMLP) and cell-free systems (H2O2/HOCl). After C. albicans and fMLP stimulation, significant concentration-dependent LACL inhibition was observed with bisabolol concentrations ranging from 7.7 to 31 mg l−1 and 3.8 to 31 mg l−1, respectively. A similar effect was observed in the SIN-1 and H2O2/HOCl systems. These findings draw attention to the possible medical use of α-bisabolol as a means of improving the antioxidant network and restoring the redox balance by antagonising oxidative stress.

According to the referred data, the potential antioxidant activity of chamomile extracts and also of its individual constituents has been substantially investigated in vitro. The study of antioxidants is an actual issue as they play an important role in the prevention of cellular damage caused by free radicals that result in several human degenerative diseases such as cancer, heart diseases, and others. The study of this potential antioxidant activity in vitro is a first approach for its extension to in vivo studies in order to evaluate the real concentrations that guarantee this effect in humans.

2.1.3. Anti-malarial activity

Nerolidol was reported as interfering with malaria growth by inhibiting glycogen biosynthesis (Lopes et al., 1999). van Zyl et al. (2006) studied the biological activity of 20 chemical standards, including the sesquiterpenoids nerolidol and α-bisabolol. A chloroquine-resistant strain of Plasmodium falciparum (FCR-3) was continuously maintained in vitro and synchronized using 5% α-sorbitol. The anti-malarial activity was determined using the triated hypoxanthine incorporation assay. The concentration that inhibited 50% of the parasite growth was determined from the log sigmoid–dose response curve and quinine was used as the reference anti-malarial agent. The 20 substances tested were found to inhibit the growth of P. falciparum with IC50 values ranging from 0.20 to 334 mg l−1 and nerolidol was the compound that exhibited the highest activity (IC50 = 0.20 mg l−1), even higher than quinine (IC50 = 23 mg l−1). α-Bisabolol also exhibited anti-malarial activity, with an IC50 = 68 mg l−1, although not as high as nerolidol. This result suggests that the observed effect is structure-dependent, and these two compounds are structurally different, as nerolidol presents an aliphatic structure, and α-bisabolol exhibits a cyclic structure combined with an aliphatic side chain (Fig. 1).

2.1.4. Anti-mutagenic activity

Gomes-Carneiro et al. (2005) investigated the mutagenicity of α-bisabolol in Salmonella microsome assay. Mutagenicity of α-bisabolol was evaluated with TA100, TA98, TA97a, and TA1535 Salmonella typhimurium strains, with and without the addition of a mixture of lyophilized rat liver (S9 mixture). Results obtained indicated that α-bisabolol do not present mutagenic activity. For the anti-mutagenicity assays, α-bisabolol was tested up to the highest nontoxic dose (i.e. 50 and 150 μg per plate, with and without S9 mixture, respectively) against direct-acting (sodium azide; SA; 4-nitroquinoline-N-oxide: 4-NQNO; 2-nitrofluorene: 2-NF; and nitro-o-phenylenediamine: NPD) as well as indirect-acting (cyclophosphamide; CP; benzoxalo-α-pyrene: BAP; aflatoxin B1: AFB1; 2-aminothracene: 2-AA; and 2-aminofluorene: 2-AF) mutagens. α-Bisabolol did not alter the mutagenic activity of SA and NPD but, at the highest dose tested (50 μg per plate), showed an inhibitory effect lowering 40% on the mutagenicity induced by 4-NQNO (1.0 μg per plate) and 2-NF (2.5 and 1.0 μg per plate). The mutagenic effects of AFB1 (0.06 μg per plate), 2-AA (0.5 μg per plate), BAP (2.5 μg per plate), 2-AF (5.0 μg per plate), and CP (20 μg per plate) were attenuated and reduced by the highest dose of α-bisabolol (100 and 150 μg per plate) by 87, 84, 80, 57, and 52%, respectively. These results indicate that α-bisabolol inhibits the effects of aflatoxin B1 and of other indirect-acting mutagens. It was also found that in rat liver microsomes α-bisabolol inhibited pentoxycresorufin-depentylyse (PRD, IC50 = 0.61 mg l−1) and ethoxyresorufin-o-deethylase (EROD, IC50 = 7.49 mg l−1), which are markers for cytochrome P450 isozymes, i.e., CYP2B and CYP1A subfamilies, a group of enzymes that use iron to oxidize potentially harmful substances by making them more water-soluble. Since CYP2B converts aflatoxin B1 and CP into mutagenic metabolites, and CYP1A activates Bop, 2-AA and 2-AF, results suggest that α-bisabolol-induced anti-mutagenicity could be mediated by an inhibitory effect on the metabolic activation of these promutagens. These results demonstrated that α-bisabolol has potential to inhibit the action of several mutagenic agents.

2.1.5. Interference on drug metabolism

Ganzera et al. (2006) investigated the inhibitory effect of chamomile essential oil on four selected human cytochrome P450 enzymes (CYP1A2, CYP2C9, CYP2D6, and CYP3A4). Essential oil (5 mg l−1) demonstrated inhibition of each one of these enzymes, and CYP1A2 was more sensitive than the other isozymes. Three constituents of the essential oil, namely chamazulene (1.40 mg l−1), cis-spiroether (1.39 mg l−1), and trans-spiroether (1.61 mg l−1) showed to be potent inhibitors of this enzyme, and also active towards CYP3A4, CYP2C9 and CYP2D6. Only chamazulene and α-bisabolol revealed a significant inhibition of the CYP2D6. This study demonstrated that the essential oil of chamomile may have the potential to modulate the metabolism of certain co-administered drugs, as it contains compounds that may inhibit the activities of major human drug metabolizing enzymes (Ganzera et al., 2006; Rodriguez-Fragoso et al., 2008). Further, α-bisabolol has already been reported in anti-mutagenic activity (Section 2.1.4) as a compound that also interferes with cytochrome P450, suggesting that this compound may underlie the metabolism of drugs and mutagenic agents.

2.1.6. Anti-platelet activity

The effect of aqueous extracts of several aromatic plants, including chamomile (100 g infusions per l of water), on human platelet aggregation has been evaluated. From among all the plants studied only chamomile, nettle, and alfalfa exhibit significant anti-platelet activity. Aqueous extracts of chamomile inhibit ADP- and collagen-induced platelet aggregation. Chamomile inhibited platelet aggregation induced by ADP (60%) and collagen (84%) as well as the whole blood aggregation induced by collagen (30%), when compared to controls (p < 0.05). Chamomile strongly inhibits thromboxane B2 synthesis induced by either ADP or collagen (Pierre et al., 2005).

2.1.7. Anti-chemotactic effect

Recent studies investigated the ability of the essential oil (Presibella et al., 2006) and aqueous-ethanolic extract (Presibella et al., 2007) prepared from the infusions of M. recutita L. to interfere with human leukocyte chemotaxis (e.g., the movement of leukocytes in reaction to a chemical stimulus) induced by casein, using the in vitro Boyden system. The analysis of the essential oil by GC–MS revealed the presence of β-farnesene, spathulenol, β-eudesmol, α-bisabolol, bisabolol oxides A and B, and chamazulene. The effects of exposing human leukocytes from peripheral blood to concentrations ranging from 0.1 to 1000 mg l−1 of chamomile essential oil, before inducing them to migrate towards a casein
gradient, resulted in an increased dose-related number of cells retained in the upper chamber compartment in comparison with the untreated control up to 10 mg l⁻¹, at which concentration it reached maximum significance, with only 66% (n = 15; p < 0.005) of the cells recovered from the lower chamber. The results showed a striking dose-related inhibition of the casein-induced human leukocyte migration after chamomile essential oil treatment in comparison with untreated controls (Presibella et al., 2006). The same results were observed for the chamomile aqueous-ethanolic extract, using the same range of concentrations (0.1–1000 mg l⁻¹) (Presibella et al., 2007). These results suggest that the exposure of human leukocytes to chamomile is associated with the destruction of leukocyte functions. These studies demonstrated that both essential oil and aqueous-ethanolic extract of chamomile may have the potential to interfere with human leukocyte chemotaxis, as they contain compounds that may inhibit the casein-induced human leukocyte migration.

2.1.8. Osteoporosis prevention

In order to research the chamomile potential as estrogen receptor modulators (SERMs – therapeutic agents), Kassi et al. (2004) studied the effect of aqueous extracts (10–100 mg l⁻¹) in a series of in vitro biological assays. Their ability to: (i) stimulate the differentiation and mineralization of osteoblastic cell culture; (ii) induce, like antiestrogens, the insulin growth; and (iii) inhibit cell proliferation of cervical adenocarcinoma cells was examined. The results demonstrated that the aqueous extracts of chamomile at the concentrations under study induced osteoblastic cell differentiation and exhibited anti-estrogenic effect on breast cancer cells without proliferative effects on cervical adenocarcinoma cells. These data open the possibility to design functional foods based on the use of chamomile aqueous extracts in order to prevent osteoporosis.

2.1.9. Anti-inflammatory activity

Chamazulene showed anti-inflammatory activity, as according to Safayhi et al. (1994), chamazulene inhibits the formation of leukotriene B4 (LTB4) in intact cells and in the supernatant fraction in a concentration-dependent mode. Chamazulene (IC50 = 0.37 mg l⁻¹) blocks the chemical peroxidation of arachidonic acid. LTB4 is a product of the 5-lipoxygenase pathway of arachidonic acid metabolism, and is a potent chemotactic factor for neutrophils. It has been postulated that chamazulene has an important role in a variety of pathological conditions including rheumatoid arthritis, psoriasis, and inflammatory bowel disease. These results are of interest as a focus on the effect of sesquiterpenic compounds on the immune system response associated with, for instance, the inflammatory process. Leukocyte activation, characterized by the respiratory burst, is an essential part of host defense against invading microorganisms. The respiratory burst leads to the formation of toxic oxidants, but since toxic oxidants cannot just be directed to kill microorganisms, the process of leukocyte activation must be strictly regulated. On the other hand, a systemic inflammation process and sepsis may lead to an exaggerated leukocyte response to stimuli and increased production of toxic oxidants, thus contributing to tissue damage and organ injury (Pascual et al., 1997). Thus, better understanding and control of leukocyte activation during inflammation and sepsis may help identify and select novel pro- or anti-inflammatory drugs, for example, sesquiterpenic compounds of M. recutita L. Moreover, as chamazulene is a compound present in the essential oil and also in chamomile aqueous-ethanolic extracts, all presenting effects on leukocyte chemotaxis, as reported in anti-chemotactic effect (Section 2.1.7), this compound might be related to the mechanism of leukocyte regulation during the inflammation process.

2.1.10. Anti-cancer effect

The anti-cancer effect of α-bisabolol and bisabolol oxide A was studied on human and rat glioma cell lines. α-Bisabolol was found to have a time- and dose-dependent cytotoxic effect on these cell lines (Cavalieri et al., 2004). After 24 h of treatment with 0.56–0.78 mg l⁻¹ of α-bisabolol, the cells’ viability was reduced by 50% compared to untreated cells. Glioma cells treated with higher concentration of α-bisabolol (2.22 mg l⁻¹) resulted in a 100% cell death. Furthermore, the viability of normal glial cells was not affected by treatment with α-bisabolol at the same concentrations as above. Since glioma is among the worst cancers against which no efficient and non-toxic treatments have so far been reported, the results obtained with α-bisabolol (no toxicity observed in cell lines and its fast accumulation in the brain), make the use of this substance very promising for the clinical treatment of this highly malignant cancer (Cavalieri et al., 2004). Ogata et al. (2010) study the cytotoxic effect of bisabolol oxide A. Bisabol oxide A was cytometrically examined on rat thymocytes by using appropriate fluorescent dyes. When the cells were incubated with bisabolol oxide A for 24 h, at concentrations of 7.15 mg l⁻¹ and 23.8 mg l⁻¹, significantly increased populations of dead cells (12.7% and 17.8% respectively), shrunken cells (39.4% and 68.4% respectively), and cells with phosphatidylserine exposed on membrane surface (33.1% and 59.3% respectively) were observed. Both cell shrinkage and externalization of membrane phosphatidylserine are general features at an early stage of apoptosis. In addition, bisabolol oxide A significantly increased the population of cells containing hypodiploid DNA (25.5% at 7.15 and 39.8% at 23.8 mg l⁻¹ of bisabolol oxide A) (DNA with a chromosome number that is one or more lower than the normal haploid number of chromosomes characteristic for a species). This increase was completely attenuated by Z- VAD-FMK, a pan-inhibitor for caspases (cysteine proteases, which play an essential role in apoptosis, necrosis and inflammation), indicating an involvement of caspase activation. These studies demonstrated that both α-bisabolol and bisabolol oxide A may have cytotoxic effects, although the reported concentrations that may reduce the viability of malignant cells were significantly higher for bisabolol oxide A than for α-bisabolol.

All these studies reported in vitro are very hopeful in order to valorise and consequently enhance the use of M. recutita L. with medicinal proposes. However, their extension to in vivo studies to assess the authentic effects in humans is essential.

2.2. In vivo studies

2.2.1. Animal model studies

2.2.1.1. Anti-inflammatory effect. The anti-inflammatory effect of aqueous and ethanolic extracts of chamomile was evaluated. In Wistar albino rats, the aqueous extract of chamomile suppressed both the inflammatory effect and leukocyte infiltration induced by a simultaneous injection of carrageenan and prostaglandin E1. The inhibitory effects of chamomile aqueous extracts were similar to those elicited by 10 mg kg⁻¹ of the anti-allergenic agent oxatome (Mckay and Blumberg, 2006). The similar results presented for in vivo studies that showed anti-chemotactic effects (Section 2.1.7) in chamomile essential oil and chamomile aqueous-ethanolic extracts, and also anti-inflammatory activity (Section 2.1.9) for chamazulene, support the possibility that this compound is linked to the mechanism of leukocyte activation during the inflammation process. In Swiss rats, the topical application of an ethanolic chamomile extract to the outer surface of the ear reduced edema induced by the application of a 2.5% emulsion of croton oil (oil prepared from the seeds of Croton tiglium). In this experiment, an ethanolic extract of chamomile containing 50 mg l⁻¹ α-bisabolol, 450 mg l⁻¹ of each, bisabolol oxide A and B, 400 mg l⁻¹ apigenin and its glucosides, 800 mg l⁻¹ dicycloethers, and 20 mg l⁻¹ azulenes was
applied to test animals in doses equivalent to 0.75, 0.25, and 0.08 mg of dried flowers per animal. The 0.75 mg of chamomile extract induced a reduction similar to that obtained with 0.45 mg benzydamine (26.6%), a non-steroid anti-inflammatory agent (Tubaro et al., 1984). Another study found that topical treatment with an extract of chamomile containing α-bisabolol (518 mg kg\(^{-1}\)), matricin (296 mg kg\(^{-1}\)), and apigenin (53 mg kg\(^{-1}\)), at a dose equivalent to 0.75 mg of dry product, was as effective as the reference drug, 0.60 mg benzydamine (n = 25), in preventing inflammation in rats subjected to croton oil induced edema (Della Loggia et al., 1990). As both aqueous and ethanolic extracts of chamomile presented anti-inflammatory effects, which are in accordance with the evidence of chamazulene activity observed in in vitro studies previously discussed, they can be studied in-depth in order to design future anti-inflammatory agents.

α-Bisabolol also inhibits the mouse ear dermatitis induced by noxious agents (arachidonic acid, phenol and capsaicin), suggesting that α-bisabolol may be a topical anti-inflammatory agent (Leite et al., 2011). In studies carried out with chamavolin and chamazulene carboxylic acid (Fig. 1), artefacts generated from matricin (dried inflorescences contain ca. 0.2% of matricin), demonstrated that these compounds present anti-inflammatory activity (Goeters et al., 2001; Franke and Schilcher, 2005; Ramadan et al., 2006; Oehler et al., 2009). Ramadan et al. (2006) evaluated the anti-inflammatory activity of chamazulene carboxylic acid in rat ear edema. Chamazulene carboxylic acid presents a chemical structure similar to profens (Goeters et al., 2001; Immimg et al., 2001), known as anti-inflammatory compounds that inhibit the key enzymes of prostaglandin metabolism – cyclooxygenase-1 and 2. In this study chamazulene carboxylic acid was found to be an inhibitor of cyclooxygenase-2 (50% inhibition at 13 mg l\(^{-1}\)), but not of cyclooxygenase-1 (17.4% inhibition at 13 mg l\(^{-1}\)). In another study (Oehler et al., 2009), also carried out using rat ear edema, chamavioline acted as an inhibitor of cyclooxygenase-2 (57% at 0.23 mg l\(^{-1}\)), not inhibiting cyclooxygenase-1 up to a concentration of 2.3 mg l\(^{-1}\). These results suggest that both compounds, chamazulene carboxylic acid and chamavioline, act as inhibitors of cyclooxygenase-2. However, chamavioline presents higher activity (57% at 0.23 mg l\(^{-1}\)) than chamazulene carboxylic acid (50% at 13 mg l\(^{-1}\)).

2.2.1.2. Anti-genotoxic effect. The inhibitory effect of chamomile essential oil on the sister chromatid exchanges produced by daunorubicin (antineoplastic, inducer of the free radicals, producing several types of genotoxic damages) and methyl methanesulfonate (alkylating agent that forms covalent bonds with DNA and modifies guanine residues causing genotoxic damage) in rat bone marrow cells was evaluated by Hernández-Ceruelos et al. (2002). Rats injected with 5, 50, and 500 mg kg\(^{-1}\) chamomile essential oil plus 10 mg kg\(^{-1}\) daunorubicin had 26%, 63%, and 76% fewer sister chromatid exchanges (SCE), respectively, than rats treated with daunorubicin alone. A similar effect was found in rats treated with 250, 500, and 1000 mg kg\(^{-1}\) chamomile essential oil plus 25 mg kg\(^{-1}\) methyl methanesulfonate had inhibition of SCE at 25%, 46%, and 63%, respectively, when compared with the control group treated with methyl methanesulfonate alone. The analysis of the essential oil revealed the presence of α-bisabolol and its oxides, chamazulene, α- and β-farnesene, germacrene D, among other sesquiterpenic compounds. These results are in accordance with those previously discussed with regard to the in vitro studies, showing that chamomile essential oil represents a source of potential anti-mutagenic agents.

2.2.1.3. Hypo-cholesterolemic effect. An aqueous extract of chamomile reduced the cholesterolemic level in hyperlipidemic Wistar rats. After 10 days, the hyperlipidemic rats treated orally

with a 6% aqueous extract of chamomile (4 mg kg\(^{-1}\)) exhibited significant decrease of serum cholesterol when compared with the control rats and no changes have been observed in serum triglyceride levels. The aqueous extract of chamomile had no effect on the control rats with respect to either cholesterol or triacylglycerides levels (Mckay and Blumberg, 2006).

2.2.1.4. Gastrointestinal effects. Several studies analysed the gastrointestinal effects of aqueous extracts and essential oil of chamomile and α-bisabolol chemical standard. When compared to papaverine, a smooth muscle relaxing drug, α-bisabolol was 91% more effective on spasms induced with barium chloride, while bisabolol oxides A and B were 46–50% more effective (Mckay and Blumberg, 2006). These results demonstrated that the aqueous extracts and essential oil of chamomile, due to the presence of compounds such as α-bisabolol and its oxides, have the potential to be effective in relaxing spasms. Bezerra et al. (2009) also investigated the effects of chamomile aqueous extracts and α-bisabolol against gastric damage induced by ethanol (96%, 1 ml per animal) in rats. These effects were assessed by determining the changes of the gastric lesion area. Chamomile aqueous extract reduced gastric damage with all doses tested (100, 200, and 400 mg kg\(^{-1}\)) and α-bisabolol at oral doses of 50 and 100 mg kg\(^{-1}\) markedly attenuated the gastric lesions induced by ethanol to the extent of 87% and 96%, respectively. Another study carried out on rats demonstrated that α-bisabolol inhibits the development of gastric ulcers induced by indomethacin, stress, and alcohol (Mckay and Blumberg, 2006). α-Bisabolol also reduced healing times in ulcers induced by either chemical stress or heat coagulation (Mckay and Blumberg, 2006). Torrado et al. (1995) studied the gastrotoxic influence of acetylsalicylic acid mixed with α-bisabolol on rats. At a dose of 200 mg kg\(^{-1}\) acetylsalicylic acid, 0.8–80 mg kg\(^{-1}\) α-bisabolol showed a significant (p < 0.05) protective effect on gastric mucosa. Also, Rocha et al. (2010), evaluate the gastroprotective action of α-bisabolol on ethanol and indomethacin-induced ulcer models in rats, and further investigate the pharmacological mechanisms involved in this action. The oral administration of α-bisabolol at a concentration of 100 and 200 mg kg\(^{-1}\) was able to protect the gastric mucosa from ethanol (0.2 ml per animal) and indomethacin-induced ulcer (20 mg kg\(^{-1}\)). The dosage of gastric reduced glutathione levels showed that ethanol and indomethacin reduced the content of non-protein sulfhydryl (NP-SH) groups, while α-bisabolol significantly decreased the reduction of these levels on ulcer-induced mice, but not in ulcer-free mice. All the studies reported above demonstrate that α-bisabolol presents very interesting gastrointestinal protective effects but the real effect on humans should be further explored.

2.2.1.5. Drug metabolizing effects in liver. The effects of chamomile infusion on the activity of hepatic phases I and II metabolizing enzymes were studied using rat liver microsomes. In this experiment, a group of Wistar rats had free access to a tea solution (2%) for 4 weeks while the control group had water (Maliakal and Wawwimolruk, 2001). The activity of phase I CYPIA2 decreased significantly whereas the activity of phase II detoxifying enzyme UDP-glucuronosyl transferase increased more than twice in comparison to the control. This study indicates that chamomile infusions can modulate the activity of hepatic cytochrome P450, and suggests that they can cause modulation of phases I and II drug metabolizing enzymes, as reported by in vitro studies of anti-mutagenic activity (Section 2.1.4) and interference on drug metabolism (Section 2.1.5) when chamomile-related sesquiterpenic compounds were used.

2.2.1.6. Central nervous system effects. A study carried out on ovarectomized rats reported that the inhalation of chamomile
essential oil reduced induced stress by increasing adrenocorticotropic hormone (ACTH) levels in plasma. ACTH is a polypeptide tropic hormone that regulates the activity of various endocrine glands, produced and secreted by the anterior pituitary gland. It is an important component of the hypothalamic–pituitary–adrenal axis and is often produced in response to biological stress, along with corticotropin-releasing hormone from the hypothalamus. Diazepam, co-administered with the essential oil, reduces ACTH levels, while flumazenil (benzodiazepine antagonist) blocks the effect of chamomile oil on ACTH. The compounds present in extracts of chamomile can also bind benzodiazepines and γ-aminobutyric acid (GABA) receptors (receptors that respond to the neurotransmitter GABA, the chief inhibitory neurotransmitter in the vertebrate central nervous system) in the brain and are thought to be responsible for some sedative effects (Mckay and Blumberg, 2006).

2.2.2. Human studies

2.2.2.1. Autonomic nervous system effects. Effects of chamomile infusions on autonomic nervous system parameters including heart beat rate, peripheral skin temperature, and mood, were examined in young Japanese males. Hot water was used as control. After chamomile infusion consumption, a decrease in heart beating rate, ratings of depression, and “sadness and depression” (determined by electroencephalography by emotion spectral analysis system) was observed, as well as an increase in skin temperature. The results demonstrate that chamomile infusion has a relaxing effect (Nakamura et al., 2002), which are in accordance with the referred results discussed with regard to the animal models studies for chamomile essential oil. This work reports an interesting biological effect associated with the consumption of chamomile infusion but more experimental assays should be carried out in order to understand the observed effect.

2.2.2.2. Hemodynamic effects. Hemodynamics comprises all the mechanisms involved in blood circulation through the cardiovascular system. The hemodynamic effects of chamomile infusions were examined in an open study of 12 heart disease patients hospitalized for cardiac catheterization. Patients had a small but significant increase in mean brachial artery pressure (91–98 mm Hg) 30 min after drinking two cups of chamomile infusion. No other significant changes in cardiac function have been observed, although 10 of the 12 patients fell into a deep sleep within 10 min of consuming the infusion (Gould et al., 1973). No explanations have yet been given to support these effects.

2.2.2.3. Topical effects. Several studies reported the topical effects of chamomile cream on skin inflammation associated with atopic dermatitis or eczemas (Hempel et al., 1999; Patzelt-Wenczler and Ponce-Pochl, 2000), radiation therapy (Maiche et al., 1991) and erythema (Korting et al., 1993). Chamomile cream has been reported to be as effective as 0.25% hydrocortisone and non-steroidal anti-inflammatory agents (Albring et al., 1983). Topical effects of chamomile cream have also been examined for its effectiveness on wound care (Mckay and Blumberg, 2006). In another study, 14 patients with weeping wounds following dermabraision for tattoo removal were treated with a compress containing chamomile extract. A significant decrease of wound area and increase of drying wound have been observed (Mckay and Blumberg, 2006). The evaluation of the effects related to the topical application of chamomile cream and compresses containing chamomile extracts were carried out based on the observation of symptoms. However, there is no information available about the cream and extract composition, nor about the relation between compound versus observed effect.

2.2.2.4. Gastrointestinal effects. The effects of chamomile-containing oral rinse on oral mucositis or stomatitis induced by cancer therapies have been examined in a clinical trial. Results showed both prophylactic (n = 66 patients) and therapeutic (n = 32 patients) effects of chamomile mouthwash in a case series of 98 head and neck cancer patients treated with either radiation or systemic chemotherapy. After the treatment with chamomile oral rinse, in the prophylactic group, one of the 20 radiation therapy patients developed grade 3 mucositis in the final week of treatment, while 13 developed intermediate-grade and 6 developed low-grade mucositis. Of the 32 patients in the therapeutic treatment group (16 radiation, 16 chemotherapy), all experienced immediate relief from mouth discomfort and within one week nearly all patients had no clinical signs of mucositis. These results suggest that the resolution of mucositis was accelerated by chamomile oral rinse (Mckay and Blumberg, 2006; Rodriguez-Fragoso et al., 2008).

3. Potential health benefits of M. recutita L.: actual knowledge and future outlook

Several health benefits have been reported for M. recutita L. essential oil and its extracts; however, a deep knowledge concerning the chemical components of chamomile responsible for each specific effect, as well as the mechanisms behind each effect, have not yet been established. Besides, in most cases, a chamomile extract standardized as to its chemical constituents is not yet available, making it difficult to compare results and to draw more realistic conclusions.

Chamomile-related sesquiterpenic compounds seem to be involved in a network of biological effects, i.e. they may exhibit themselves specific health benefits, may have synergic effects with drugs such as antibiotics, and/or may underlie the metabolism of certain co-administered drugs and mutagenic agents. It is important to point out that the quantities necessary to have a biological effect, i.e., the dose is effect-dependent, and for the studies reported above, the doses are in the order of mg l⁻¹. However, depending on the structure of the compound and on the biological effect, these can be as low as mg l⁻¹. When chamomile extracts, i.e., a mixture of several chemical structures, were tested, the minimum concentration to have an effect can be higher.

The anti-microbial, antioxidant, anti-malarial, anti-mutagenic, anti-inflammatory, and anti-cancer activities are explained by the presence of sesquiterpenic compounds (Safayhi et al., 1994; Rekka et al., 1996; Lopes et al., 1999; Brehm-Stecher and Johnson, 2003; Cavalieri et al., 2004; Gomes-Carneiro et al., 2005; van Zyl et al., 2006; Simões et al., 2008). On the other hand, anti-chemotactic, anti-inflammatory, anti-genotoxic, and gastrointestinal activities were tested using aqueous and ethanolic extracts or essential oil of M. recutita L. (Hernández-Ceruelos et al., 2002; Gonzera et al., 2006; Mckay and Blumberg, 2006; Presibella et al., 2006; Rodriguez-Fragoso et al., 2008), which contain sesquiterpenic compounds among other components. Sesquiterpenic compounds present a low solubility in water but the amount that can be solubilised seems to be enough to promote their transference from the plant to the water. For example, the solubility in water for α-bisabolol, β-farnesene, farnesol, and nerolidol are, respectively, 0.0023, 0.0108, 1.7, and 2.07 mg l⁻¹, at 25 °C (Syracuse Research Corporation, 2010). Furthermore, the solubility increases with temperature, and usually the aqueous extraction occurs at a temperature higher than room temperature (ca. 70 °C). Nerolidol is usually reported as a constituent of chamomile aqueous extracts and exhibits anti-malarial activity at a concentration ten times lower (0.2 mg l⁻¹) than its solubility in water (van Zyl et al., 2006).
These results clearly suggest that in order to valorise chamomile, several approaches may be improved. One of them should include the development of efficient and solvent-free methodologies to obtain a sesquiterpenic-enriched extract like, for example, supercritical fluid extraction (Povh et al., 2001) for the specific evaluation of their biological effects. Another approach should be to plan in vivo studies using animal models, in order to in-depth study the mechan-

4. In vitro and in vivo studies: drawbacks and future trends

This work gives data that open several opportunities for the valuation of chamomile raw material, by looking for the potential effects of its components, including the sesquiterpenic compounds. However, the lack of human trials to confirm the potential health benefits associated with chamomile components may bring some limitations to the valuation of this medicinal plant. Consequently, in vivo studies are essential to evaluate the safety of several natural products to which people may be exposed. Unfortunately, in vivo studies have a number of drawbacks: (1) ethical issues surrounding animal experimentation, namely, animal suffering and induction of diseases such as obesity, diabetes or cancer; (2) the need to extrapolate from animal to humans in terms of physiology, biochemistry, genetics and behaviour; (3) the economic costs of long-term studies; and (4) the possibility of missing idiiosyncratic human reactions. To predict human in vivo responses based on in vitro studies, computational methods for prediction of hazards and determination of mechanistic information about the compounds under study are required. A dynamic model of the human upper gastrointes-

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