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R Soulimani
Université de Lorraine, LCOMS/ Neurotoxicologie Alimentaire ET Bioactivité, Campus Bridoux, Metz, France

J Bouayed
Université de Lorraine, LCOMS/ Neurotoxicologie Alimentaire ET Bioactivité, Campus Bridoux, Metz, France

Rakesh Kumar Joshi
Lecturer Chemistry, Department of Education, Government of Uttarakhand, India

Correspondence:
R Soulimani
Université de Lorraine, LCOMS/ Neurotoxicologie Alimentaire ET Bioactivité, Campus Bridoux, Metz, France

Limonene: Natural monoterpene volatile compounds of potential therapeutic interest

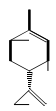
R Soulimani, J Bouayed and Rakesh Kumar Joshi

Abstract
The majorities of essential oils are extracted from aromatic and essential plants and have been used in their natural state for millennia for many applications. They consist of bioactive ingredients (terpenes) known for many biological properties. Their physicochemical characteristics classify them as volatile and fragrant natural organic compounds (FOCs) and play an important role in Plante-Animal communication, in the defense of plants and forests against natural aggressions, but also to fight against drought by contributing rain. It is important to distinguish FOCs from VOCs (volatile organic compounds) synthesized or produced from petrochemical derivatives or from purified natural compounds and have real potential adverse effects. We carried out this review to list the different works carried out on the essential oils rich in one of the most known and used natural compounds (Limonene) in order to highlight its beneficial properties and its possible harmful effects by specifying the various conditions of studies. It is also important to emphasize the difference between volatile organic contaminants (VOCs), which are generally pollutants resulting from human activity or the synthesis or purification of molecules, and fragrant organic compounds (FOCs), whose ratio benefit risk is more in favor of their use with a good control of the recommendations on the doses of use.

Keywords: VOCs, (FOCs), limonene, pharmacology, toxicology, terpenes, risk/benefit

1. Introduction
Limonene is a ubiquitous terpene that is found, among others, in essential oils of lemon tree, orange, neroli, bergamot, and tangerine. Limonene is one of the major constituents of citrus peel, and its presence in the citrus peel contributes to their smell. Limonene is a monoterpene formed from two isoprene units, which are five-carbon molecular building blocks. Limonene exists as two enantiomers, d-limonene and l-limonene. The more common d-enantiomer has a strong smell of oranges; however, in the presence of aird-limonene can be slowly oxidized [1]. For industrial purposes, d-Limonene is recovered from citrus fruits through two primary processes namely steam distillation and centrifugal separation. It is commonly used as a base ingredient in the manufacture of cleaning products as a solvent; and is also used in chemical synthesis as a *p*-cymene carvone precursor [2]. Natural limonene has a pleasant more like lemon scent, making it widely used as a flavor and fragrance additive in common foods, such as fruit juices, candies, chewing gums, soft drinks and ice creams. Limonene is one of the most common fragrances used in the formulation of cosmetics, and can be found in many types of beauty products such as soaps, perfumes, shampoos, hair conditioners and shower gel, cleaners and biocides. In addition, limonene is considered safe for food preservation and could be used as a green solvent for the extraction of natural products. Limonene can be also of synthetic origin. Since the 1990s, the annual world production of d-limonene and orange essential oil (95% d-limonene) has exceeded 45 kilotons. This molecule plays an important role in the composition of these essential oils and helps to reduce the irritating action of citrals (also present in these essential oils).

2. Physico-chemical properties
The molecular structure of d-limonene consists of 10 carbon atoms and 16 hydrogen atoms: ($C_{10}H_{16}$).



The following table presents several physicochemical properties of d-limonene

Chem. Abstr. Name	®-1-Methyl-4-(1-methylethenyl) cyc1ohexene
Synonyms	Cajaputene; carvene; cinene; (+)-dipentene; d-(+)-limonene; D-(+)-limonene; (+)-limonene; ®-limonene; ®-(+)-limonene; (+)-para-mentha-1,8-diene; ®-(+)-para-mentha-1,8-diene; 1-methyl-4-isopropenyl cyc1ohexene-1; Refchole
Description	Colourless liquid
Melting point	-74.3 °C
Boiling point	175.5-176 °C
Density	0.8411 g/cm3 at 20 °C/4 °C
Solubility	insoluble in water; soluble in benzene, carbon tetrachloride, diethyl, ether, ethanol and petroleum ether, soluble in glycerine
Optical rotation	~ + 125.6°
Spectroscopy data	Infrared, nuclear magne tic resonance and mass spectral data have been reported
Stability	Oxidizes to film in air

3. Production

3.1 Manufacturing process

D-Limonene is widely distributed in nature. It has been detected in more than 300 essential oils [3]. D-Limonene is a by-product of the orange juice, lemon and grapefruit industry [4]. It is obtained from the peel oil of these citrus fruits in which its concentration can reach up to 97% by weight. Recovery of d-limonene is by conventional extraction using two methods. The brown oil extractor ("Brown Oil Extractor") recovers the oil before extraction of the juice while the FMC online extractor ("FMC in Line Extractor") recovers fruit oil during the juice extraction process [5, 6]. Both processes function by mechanical breakage of the cells containing the essential oil, located in the fruit's epicarp. An aqueous emulsion is thus obtained; the essential oil is then separated by centrifugation.

3.2 Toxicity, absorption and metabolism

3.2.1 Animal exposure

A. Toxicity in animals

LD50s of oral d-limonene in mice and rats are generally exceeding 5g/kg body weight. The dermal LD50 in the rabbit is also greater than 5g/kg body weight. Intraperitoneal LD50s range from 1.3g/kg and 4g/kg in mice and rats, respectively. The intravenous LD50 in rats is 0.1g/kg (body weight) [7-11].

B. Animal's effects

1. Liver

Studies on the hepatic effects of d-limonene in animals are particularly relevant as they have served as a basis for the establishment of exposure limit value guidelines by institutions such as the American Industrial Hygiene Association (AIHA) and Sweden [12-14]. The various effects include increases in liver weights in rats as well as dogs, and the appearance of multi-nucleus cells and cytomegaly in male mice. In addition, in some studies, an increase in the amount of cytochrome P450 and the activity of several hepatic enzymes, as well as the bile flow in the rat have been observed [7, 15-17].

2. Carcinogenicity

To evaluate its carcinogenic potential, the US National Toxicology Program (NTP) has conducted a chronic toxicity study of d-limonene by gavages in two animal species. Male rats (n=50) were administered d-limonene 5 days per week for 103 weeks at the doses of 0, 75 and 150 mg per kg of body weight; however, female rats received the doses of 0.30 and 600mg/kg body weight. In addition, male mice (n=50) received d-limonene doses of 0, 250 and 500mg/kg body weight while female mice were administered doses of 0, 500 and 1000mg/kg body weight. NTP reported only in male rats a significant dose-dependent increase in renal tubular cell hyperplasia, adenomas, and adenocarcinomas [18]. (US-NTP, Testing Status of D-Limonene, 1990 https://ntp.niehs.nih.gov/testing/status/agents/ts-10071-t.html) Nevertheless, other studies conducted by Crowell and Gould [19], Sun *et al.* [20] and Kim *et al*., [11] reported that d-limonene prevents the appearance of various types of cancers in rodents and even evokes its therapeutic use for tumors in humans.

3. Reprotoxicity and developmental toxicity

Developmental studies demonstrated that oral prenatal exposure to d-limonene at 500 and 2900mg/kg from 9th to 15th day of gestation in rats, at 500 and 2400 mg/kg from 7th to 12th day of gestation in mice) and to 250, 500 and 1000 mg/kg from 6th to 18th day of gestation in rabbits, that there was an absence of teratogenic or foetotoxic potential [9, 10, 21]. The survival rate of frog embryos (*Xenopus frog* L.) exposed to up to 114 ppm d-limonene in water has not been affected by this treatment [9, 10, 21], which was in keeping with studies of Kodama [22, 23] and Tsuji *et al.* [24], and the report of FAO/ WHO [7]. D-Limonene is virtually non-toxic when administered subacutely in the quail (*Coturnix coturnix* L.) diet. Indeed, a lethal concentration 50 (LC50) is higher than 5620 ppm of d-limonene in this bird. D-Limonene is slightly toxic when given to trout, such as an LC50 of 80 ppm d-limonene in this fish. D-limonene is classified as "slightly toxic" in *Daphnia* because of an LC50 equal to 39 ppm of d-limonene in this small crustacean [1].

4. Human exposure

D-Limonene is generally recognized as safe for human consumption as a synthetic flavouring substance (US Food and Drug Administration, FDA, 1991). The tolerable daily intake (TDI) of d-limonene was estimated at 0.27 mg / kg body weight, i.e., for an adult of 60 kg, the TDI is 16.2 mg (International Agency for Research on Cancer, IARC, [3]. This dose of d-limonene comes naturally from food and its addition as a flavor.

5. Toxicokinetics

1. Administration

According to the IARC limonene monograph [3], following the oral administration of 14-carbon d-limonene to two volunteers at 1.6 g, 55 to 83% of the dose was excreted in the urine in less than 48 hours. The main urinary metabolite was 8-hydroxy-*p*-menth-1-en-9-yl-β-D-glucopyranosiduronic acid. Falk-Filipsson *et al.* [25] exposed eight male volunteers, during light physical exercise, to atmospheric concentrations estimated at 10, 225 and 450mg/m^3 of d-limonene during 3 times of 2 hours spaced 2 weeks each. Pulmonary absorption, calculated as the difference between the amount of solvent in the inspired air and that in the exhaled air, was high: 68% of the dose administered for the two highest concentrations and 63% for the lowest concentration.

2. Metabolism
D-Limonene is metabolized rapidly and almost completely: the blood clearance measured up to 21 hours after exposure was stopped at 450mg/m^3 was 1.1 L/kg h.

3. Elimination
The authors determined 3 phases of blood elimination: an initial phase (0-15 min after exposure), an intermediate phase of rapid elimination (16-319 min after exposure) and a terminal phase of slow elimination (320-1300 min after exposure). The blood elimination half-lives of d-limonene were approximately 2.6 min for the initial phase, 32 min for the intermediate phase and 750 min for the terminal phase. The longer half-life of this last phase suggests accumulation in adipose tissue. About 1% of the absorbed d-limonene is eliminated unchanged in the exhaled air after the exposure has stopped. Urinary excretion of unchanged d-limonene was only 0.003% of the initial dose [22]. A male volunteer was exposed to d-limonene by immersing his hand in the solvent for two hours Falk *et al*. [25], the concentration of d-limonene in the arterial blood of the unexposed hand 20 minutes after the end of exposure was approximately 0.14 µmol/L, almost 5 times lower than in the blood of another volunteer exposed by inhalation to an atmospheric concentration of 10mg/m^3 of d-limonene for two hours. It can be concluded that for d-limonene, percutaneous absorption is relatively low in humans compared to the pulmonary route of entry. In humans, d-limonene is easily absorbed by the lungs and less so by the skin. It is excreted mainly in the urine as conjugated hydroxyl compounds. D-Limonene is a skin irritant; allergic contact dermatitis may develop when the product is degraded by oxidation. According to studies, the systemic toxicity of d-limonene is considered to be low in humans. The product is not genotoxic, teratogenic or foetotoxic [9-10, 21].

C. Clinical effect
1. Skin and eyes
A few minutes after the start of the exposure, a volunteer whose hand was immersed in d-limonene for a period of 2 hours, suffered from itching with pain and burning sensations increasing with time. The itching decreased after the end of the exposure while the burning sensation intensified for at least 10 minutes. The skin on the back of the hand was swollen and erythematous after exposure. The swelling disappeared 100 min after the exposure. Severe purpura developed 6 hours after the end of the exposure reaching its climax after 1 to 2 days and remained for several weeks [28]. The works of Karlberg *et al*. [8, 14] as well as international reports of the evaluation of chemicals [29] stressed the non-existence of reported cases of allergic contact dermatitis caused solely by the exposure to d-limonene. The works of Karlberg *et al*. [8, 14] and Kim *et al*, [10], demonstrated that the presence of auto-oxidation products (1, 2-epoxylimonene, carvone, limonene hydroperoxides) derived from pure d-limonene (synthetic product) is a necessary condition for the development of its allergenic potential. These degradation products of pure d-limonene appear following a very prolonged exposure of the solvent to the open air. However, the presence of antioxidants prevents the formation of oxidation products in d-limonene for several months, which could be in favor of its use in an essential oil with other ingredients with potential antioxidant rather only in a pure state. According to the IARC, 1998, antioxidant-free d-limonene, protected from light and air at 4 to 6 °C for one year, does not oxidize (IARC, 1998).

2. Respiratory system
Falk-Filipsson *et al*. [25] exposed 8 volunteers to d-limonene by inhalation for 2 hours at the following concentrations: 10, 225, 450mg/m^3. During the exposure, the volunteers provided equivalent physical effort. Subjects did not experience irritation of the upper respiratory tract. The authors reported a statistically significant (-2%) decrease in post-exposure lung capacity in volunteers exposed to the highest concentration compared to the lowest concentration, and they suggested that this small amplitude change has no functional significance. Other parameters such as expiratory volume, residual volume, and total lung capacity were not affected. Previous studies and reviews have confirmed these observations [30-32]. Very recent clinical work had shown no difference noted on airway inflammation, lung function or asthma control in mild and moderate allergic asthmatics after exposure twice a day for one month, to a spray containing a mixture of 41 essential oils [33-34].

3. Liver
In another study in which d-limonene (97%) was infused directly into the biliary system of patients with 5-29 ml of d-limonene in order to dissolve their biliary calculus, the authors noted an elevation of transient aspartate aminotransferase (AST), alanine aminotransferase (ALT), and serum alkaline phosphatase during infusions [35]. In contrast, another study, in which 20 g of d-limonene was orally administered to 5 male volunteers, did not cause any biochemical changes in blood such as total protein, total bilirubin, total ASAT, ALT and alkaline phosphatase in the liver [9].

4. Kidney
The authors of a Japanese study, in which 5 volunteers ingested a single dose of 20 g of d-limonene, report a low transient proteinuria [9-10]. The IARC report and several studies demonstrated that the functions of the kidneys and pancreas were unaffected by ingestion of limonene [14, 36-42].

5. Nervous system
In the study by Falk-Filipsson *et al*. [26-27] (24 a/b), when 8 volunteers were exposed, for 2 hours, to atmospheric concentrations up to 450mg/m^3 d-limonene, the subjects reported no symptoms attributable to an effect on the central nervous system. Several other studies also showed that brain function was unaffected by ingestion of limonene [43, 3, 40]. The work of Yasue Yamadaa *et al*., 2015 [44] showed that fragrant organic compounds FOCs had much lower toxicities than volatile organic compounds (VOCs) and indicate that FOCs are safer than other compounds.

6. Mutagenicity and genotoxicity
D-Limonene and its metabolites showed no activity in Ames mutagenesis tests on various strains of *Salmonella typhimurium*, with or without metabolic activation [3]. Various tests on mammalian cell cultures, notably the absence of sister chromatid exchange and chromosomal aberration in hamster ovary cells, confirm the absence of genotoxicity of this monoterpene [41, 45, 46, 3, 20].

7. Environnent
D-Limonene is a ubiquitous substance in the environment. In addition to its presence in a wide variety of foods and plant species, it has been detected in the urban and forest atmosphere, indoor air, estuarine sediments, sewage sludge

and in drinking water. If spilled on wet or dry outdoor soil, d-limonene is expected to volatilize rapidly but adsorption may attenuate this phenomenon. This evaporation should also occur during a spill into watercourses. The volatilization half-life in a river was estimated at about 4 hours. D-Limonene released to the outside atmosphere is expected to degrade rapidly by reacting with hydroxyl radicals, tropospheric ozone and overnight with nitrate radicals. The half-lives calculated for these three processes were estimated at 2 hours, 25 minutes and 3 minutes, respectively [1,47].According to IRTA [48] and USEPA [49], d-limonene is a photochemically reactive VOC. However, emission levels in atmospheric air should be low because of its low vapor pressure. (25 °C). In conclusion, for this part, low-dose d-limonene is nontoxic in humans. The work of Vieira *et al.* [50], following oral administration, showed that limonene is rapidly absorbed in the gastrointestinal tract, distributed and metabolized. Limonene is considered as a safe non-harmful compound with very low toxicity to humans, without inducing a risk of mutagenicity, carcinogenicity or nephrotoxicity. Schmidt and Göen, 2017 [51] have studied limonene metabolism and elimination kinetics in humans. The metabolism of limonene in humans occurs rapidly and the body virtually eliminates metabolites after 24 hours of ingestion of limonene. The Food and Agriculture Organization of the United Nations (FAO) and the World Health Organization (WHO) [7] recommend a maximum acceptable daily intake of 1.5mg/kg body weight for d-limonene. In the workplace, d-limonene exposure levels can be very high for short periods of time, especially when manually scrubbing metal parts in soaking tanks or when working in confined spaces. D-Limonene passes through human skin but much less than by the pulmonary route. Pure d-limonene is not allergenic. On the other hand, pure d-limonene exposed to the air oxidizes and causes allergy. The auto-oxidation products of d-limonene are responsible for the phenomenon. There is, however, no cross sensitization between the auto-oxidation products and d-limonene itself. Since 1997, Chang *et al.* [52] suggest that dermatologists include oxidized d-limonene in the patch battery for screening for the causative agent in patients with hand eczema.

4. Uses and Therapeutic Properties of Limonene

Many therapeutic properties have been attributed to and demonstrated with limonene. Many publications of the last ten years (2009-2019) have highlighted numerous therapeutic effects of natural limonene contained in essential oils.

4.1 Anti-inflammatory activity

Kummer *et al.* [53] investigated the anti-inflammatory activity of *Citrus latifolia* Tanaka fruit essential oil rich in limonene using the zymosan induction test of peritonitis and other *in-vitro* tests. The authors confirmed that limonene was the main compound present (62%) in the essential oil of *C. latifolia*. Both the essential oil and limonene did not show *in-vitro* cytotoxicity on neutrophils from the peritoneal cavity of BALB/c mice. Limonene decreased neutrophil migration when chemo-attractants were used. *In vivo*, oral limonene pretreatment reduced leukocyte infiltration as well as pro-inflammatory cytokine TNF-α levels in peritoneal exudate after zymosan-induced peritonitis in BALB/c mice. The anti-inflammatory cytokine interleukin 10 (IL-10) levels were not modified. The authors attributed the anti-inflammatory effect of limonene to reduced levels of TNF-α, and also to the reduction of chemotaxis of neutrophils and leukocytes.

Macrophages play an important role in the inflammatory response, including the overproduction of pro-inflammatory cytokines and inflammatory mediators. To study the effects of limonene on the production of cytokines and inflammatory mediators in macrophages, Yoon *et al.* [54] incubated RAW 264.7 macrophages and treated them with both lipopolysaccharide (LPS) and several concentrations of limonene. Limonene reduced the expression of inducible nitric oxide synthase (iNOS) and cyclooxygenase (COX) as well as the production of prostaglandin E2 (PGE 2), which is responsible for the inflammatory responses. Pro-inflammatory cytokines TNF-α, interleukin 1 β (IL-1β) and interleukin 6 (IL-6) levels also decreased after treatment with limonene, in a dose-dependent manner. The authors therefore suggested that limonene could be an effective anti-inflammatory agent for use as a cosmetic or drug in skin disorders. Doxorubicin is a drug used for the treatment of cancer and has side effects such as the production of reactive oxygen species (ROS) as well as increasing the inflammatory response, causing tissue damage. According to Rehman *et al.* [55], limonene could protect the kidneys from the side effects of doxorubicin. Prior to administration of doxorubicin, limonene was included in the diet of Wistar rats for 20 days. Pretreatment with limonene resulted in decreased levels of pro-inflammatory markers. Limonene also reduced the damaging action of doxorubicin in the kidneys, exhibiting antioxidant activity, restoring glutathione and superoxide dismutase levels, and increasing catalase, glutathione peroxidase, and glutathione transferase activities.

According to this work (Rehman *et al.* [55], renal injury induced by doxorubicin was attenuated by a dual anti-inflammatory and antioxidant activity of limonene, indicating that this compound could be proposed in combination with doxorubicin as a complementary therapy. Osteoarthritis is a degenerative disease, characterized by joint inflammation and loss of cartilage, among other complications. Rufino *et al*. [56] tested the activity of limonene in human chondrocytes stimulated by IL-1β, a cellular model of osteoarthritis. IL-1β-induced nitric oxide (NO) production and inflammatory markers, anti-catabolic and pro-anabolic gene expression were evaluated. Limonene, at three different concentrations, inhibited NO production and activation of NF-κB and p38. Similarly, limonene decreased the expression of inducible nitric oxide synthase (iNOS), in addition to metalloproteinase-1 (MMP-1) and metalloproteinase-13 (MMP-13), which are catabolic genes coding enzymes responsible for the hydrolysis of major matrix components specific to articular cartilage. Limonene induces an increase in the expression of the anti-catabolic tissue inhibitor of metalloproteinase 1 (TIMP-1), but it is not able to increase the expression of genes specific to the extracellular matrix, such as collagen II.

Studies of the effects of limonene on HL-60 clone 15 cells of human eosinophilic leukemia, performed by Hirota *et al.* [57] showed that limonene, isolated from *Citrus junos* Tanaka fruit rind essential oil, decreased phorbol 12-myristate 13-acetate (PMA)-induced ROS production in clone 15 df-HL-60-cells, before and after stimulation with eotaxin. "Monocyte chemoattractant protein-1" (MCP-1) is a chemokine produced spontaneously by eosinophilic cells and its production increases the recruitment of leukocytes, triggering inflammatory or allergic reactions. Limonene decreased the production of MCP-1 by cells, even in the presence of a potent proteasome inhibitor. Limonene also decreased the activity of NF-κB in nuclear extracts of cells and decreased chemotaxis when combined with a specific inhibitor of p38 mitogen-activated protein kinase (MAPK). According to these

studies, limonene exhibited an anti-inflammatory effect by decreasing ROS production, NF-κB activity and eosinophil migration. Limonene has an anti-inflammatory effect *in vitro* and *in vivo* primarily by modulating the action of cytokines and by regulating pathways closely related to the inflammatory response. Orange peel extract (OPE) containing limonene has been used as a dietary supplement in a clinical study in elderly (aged 65-85) and healthy people in three countries. In this study, it was compared between people who had daily received a soft capsule containing OPE for 56 days and people without any treatment. According to the results of these studies, there was no difference between day 1 and day 56 of the treatment, but the authors established a score on inflammatory status, in which IL-6 levels were significantly decreased after 56 days of treatment. The authors concluded that limonene could be used as a dietary and therapeutic supplement in anti-inflammatory treatments [58].

4.2 Antioxidant activity

Oxidative stress is a consequence of the overproduction of ROS in the body during exposure to hostile environments and is linked to many diseases. At the level of the organism, there are antioxidant mechanisms that have the ability to eliminate ROS to mitigate their deleterious effects on the various organists, in cells, and on the various organs of the body. These endogenous antiradical effects limit or neutralize the damages caused by the overproduction of ROS. Following repeated exposure to stressors, the endogenous system of antiradical protection is overwhelmed by the overproduction of ROS. Thus, the ingestion of natural products having an antioxidant/antiradical power can contribute significantly to protect the body against the deleterious effects induced by oxidative stress. The overproduction of ROS is one of the complications that is often associated with various pathologies including diabetes mellitus in humans. Murali *et al.* [59, 60] induced diabetes using streptozotocin in male Wistar rats and treated them orally with limonene for 45 days. Treatment with limonene showed a decrease in all lipidic and enzymatic markers of oxidative stress. The studied rats showed a significant increase in superoxide dismutase, catalase, glutathione peroxidase and glutathione transferase. In addition, glutathione level and vitamin C activity were higher in the limonene-treated rats. Treatment with limonene also prevented lesions observed in the liver and kidneys compared to treatment with streptozotocin alone which showed tissue damages in this animal model of diabetes. Overall, these studies showed that limonene possesses *in-vivo* antioxidant activity, against oxidative stress in diabetic rats.

Similar results were observed in cultured murine lymphocytes from BALB/c mice. Low concentrations of limonene exhibited high scavenging activity against 2,2-diphenyl-1-picrylhydrazyl (DPPH), which is a synthetic free radical. In addition, increased catalase and peroxidase activity, and reduced levels of peroxide hydrogen were found. In this study, limonene at low concentrations stimulated cell proliferation. At high concentrations, limonene has shown an increase in superoxide dismutase activity. These results suggest that limonene can protect lymphocytes against oxidative stress and at low concentrations; it can also stimulate cell proliferation [61].

Oxidative stress is also associated with the development of cataracts. Bai *et al*. [61] induced oxidative stress in human lens epithelial cells using hydrogen peroxide after treatment with limonene. Hydrogen peroxide caused a decrease in cell viability, however in cells treated with limonene an increase was observed. Limonene reduces the expression of caspase-3 and caspase-9, which are proteins related to apoptosis. The Bax/Bcl-2 ratio also plays an important role in apoptosis; limonene could prevent the reduction of this ratio caused by hydrogen peroxide. In addition, limonene inhibited the phosphorylation of p38 MAPK, which activates apoptosis induced by oxygen peroxide. These results suggested that limonene can protect epithelial cells of the lens against oxidative stress by antioxidant and anti-apoptotic pathways.

By studying the antioxidant, cytotoxic, genotoxic and antigenotoxic effects of limonene, Bacanli *et al.* [62] showed that limonene did not cause genotoxic damage in lymphocytes and fibroblasts via the comet test and decreased the frequency of micronuclei in cells. In conclusion, limonene prevented oxidative DNA damage in lymphocytes and fibroblasts.

Ahmad and Beg [63] analyzed the hypolipidemic and antioxidant effect of limonene and thymoquinone in rats exposed to an atherogenic diet. As a result, limonene demonstrated lipid-lowering efficacy and attenuated oxidative stress-induced cardiovascular disease by decreasing HMG-CoA reductase activity and restoring MDA values. In addition, limonene reduced the oxidation of low-density lipoprotein (LDL). In conclusion, limonene exhibited a potential atheroprotective and antioxidant effect in rats fed an atherogenic suspension.

4.3 Anticancer activity

Citrus bergamia essential oil, composed of 70% limonene + linalyl acetate, reduces the proliferation and survival rate of human neuroblastoma cells SH-SY5Y [64]. Russo *et al.* [65] studied the main compounds of the essential oil of *Citrus bergamia* by testing them individually in neuroblastoma cultures, in order to identify those responsible for the death of neuroblastoma cells. Exposure to the mixture of both terpenes induces mitochondrial damage, cytoskeletal reorganization, and decreased cell volume. All of these alterations were concentration-dependent and only occurred with the combined exposure of limonene and linalyl acetate to SH-SY5Y cells. By studying the antitumor effects of limonene and berberine, as well as the combination of take two, Zhang *et al.* [66] concluded that these compounds synergistically exert anti-cancer effects on the MGC803 human gastric carcinoma cell line. Limonene and berberine, when combined, increase apoptosis, ROS generation, and caspase-3 expression.

In the same way, Jia *et al.* [67] investigated limonene activity in LS174T human colon cancer cells. The researchers found that limonene, in a dose-dependent manner, decreased the viability of LS174T cells and induced apoptosis. The authors concluded that the anticancer effect of limonene was involved in the activation of the mitochondrial apoptosis signaling pathway.

Rabi and Bishayee in 2009 [68] used limonene to improve the tumor response to docetaxel in metastatic prostate cancer, using DU-145 human prostate carcinoma cells in search of strategies to improve the efficacy of treatment of docetaxel. The authors observed that limonene and docetaxel in combination improved the cytotoxicity to prostate carcinoma cells, but not normal prostatic epithelial cells, which may be explained by the fact that normal cells express more effective antioxidant mechanisms than cancer cells. The combination of docetaxel and limonene also induced ROS production by decomposing hydrogen peroxide and increasing caspase activity compared to docetaxel alone. The combination therapy also induced cleavage of caspase-9 and caspase-3, an inverted apoptotic effect after pretreatment with *N*-

acetylcysteine, a potent antioxidant, demonstrating that the antitumor effect involved the production of ROS with strong participation of caspases.

In this study by Rabi and Bishayee [68] limonene increased the antitumor effect of docetaxel against prostate cancer cells without any cytotoxicity to normal prostate epithelial cells, and this effect was mediated by ROS generation and caspase-9 and caspase-3 activation.

According to these studies, limonene has potential anticancer activity *in vitro*, but more *in vivo* studies are needed to obtain more conclusive responses to this effect.

Miller *et al.* 2013 [69] investigated the effects of limonene on breast cancer in newly diagnosed women, hypothesizing extensive distribution of the compound in breast tissue that may have preventive effects. Forty women ingested 2 g of limonene daily for 2 to 6 weeks; the average concentration of limonene in the breast tissue was 41.3 μg/g. The authors observed a decrease in the expression of cyclin D1, a protein involved in tumor cell proliferation.

4.4 Analgesic or anti-nociceptive activity

In a recent study, *Piccinelli et al.* [70-71] examined the oral administration of limonene to mice previously intrathecally injected with glycoprotein (gp120), IL-1β or TNF-α, with the hypothesis that limonene could prevent hyperalgesia caused by inflammatory cytokines or gp120. Limonene was able to decrease the production of IL-1β and IL-10 after intrathecal injection of gp120. The activity of superoxide dismutase was increased after spinal injection of IL-1β or TNF-α in limonene-treated mice, showing that limonene may be involved in the antihyperalgesic effect since superoxide dismutase plays a role in the management of pain. In conclusion, limonene prevents hyperalgesia caused by gp120, IL-1β and TNF-α, probably by modulating cytokine production and superoxide dismutase expression. Kaimoto *et al.* [72] investigated *in vivo* and *in vitro* effects of limonene on potential transient receptor (TRP) channels, which are related to pain perception. Systemic administration of limonene in mice induced a decrease in the nociception induced by hydrogen peroxide. In addition, following the treatment of DRG neurons and HEK 293 cells with hydrogen peroxide, limonene reduced the nociceptive effect of TRPA1 stimulation. With these results, the authors suggested that limonene has an inhibitory effect on TRPA1 mediated nociception. Such results suggest that it is possible to say that limonene has an anti-nociceptive and analgesic effects.Very recent clinical studies have shown the effectiveness of a mixture of 8 essential oils on headaches under real conditions in a single crisis [73].

4.5 Antidiabetic activity

Studies using leaves of *Aegle marmelos* L., an aromatic tree containing limonene as the main compound, showed antidiabetic activity. *Aegle marmelos* extract induced an antihyperglycemic effect in male Wistar rats with streptozotocin-induced diabetes [74] and significantly decreased the formation of advanced glycation products. Renal function was unaffected, so treatment could prevent complications such as nephropathy and cataracts. The study of the mechanisms of inhibition of glycation by limonene has shown that this inhibition occurs by stabilizing the structure of the protein by hydrophobic interactions. A more recent study by Joglekar *et al.* [75] evaluated the combined use of limonene and aminoguanidine, a treatment widely used in the prevention of many diseases such as certain nephropathies, atherosclerosis, cataract formation and neuropathy. The aminoguanidine-limonene combination eliminates the side effects of aminoguanidine alone. According to these studies, limonene is a potential alternative as an anti-glycating agent, when it used at low doses.

4.6 Effects on the gastrointestinal tract

Several studies have investigated the effects of gastroprotection and healing of ulcers by citrus essential oils and their main component limonene, in the exploration of new compounds without inducing side effects. A study by Moraes *et al.* [76] has demonstrated the action of limonene and *Citrus aurantium* essential oil on ethanol-induced ulcers on the gastric mucosa in rats. The results showed that the essential oil is 97% limonene. The gastroprotective effect could be explained by the increase in gastric mucus, which neutralizes the concentration of H^+ in gastric acid. In acetic acid-induced gastric ulcers, *Citrus aurantium* essential oil caused 44% and 56% cure rates, respectively, in male Wistar rats. The researchers, while inducing an increase in cell proliferation, an increase in angiogenesis and a production of prostaglandin E2, confirm the improvement of the integrity of the mucosa. Rozza *et al.* [77] studied the gastroprotection of *Citrus limon* essential oil and its major compounds, limonene and β-pinene. In the indomethacin-induced gastric ulcer model, the essential oil and limonene have provided effective gastroprotection; however, β-pinene did not show gastroprotective activity. The authors concluded that limonene was probably the only component responsible for the gastroprotective effect of *Citrus limon* essential oil, stimulating the production and secretion of mucus and heat shock protein HSP70. D'Alessio *et al.* [78-79] aimed to study the anti-inflammatory effect of limonene using a model of induced colitis in rats and cell cultures of fibroblasts and enterocytes. In the colitis model, limonene was administered to Wistar rats after injection of 2, 4, 6-trinitrobenzenesulfonic acid (TNBS) to induce colitis. Compared with ibuprofen, limonene-treated rats had decreased inflammatory scores and serum TNF-α concentration compared to untreated rats. Using cultured fibroblasts, limonene-containing orange peel extract was able to inhibit TNFα-induced NF-κB translocation, suggesting that limonene acts on the NF-κB pathway. Limonene also increased epithelial resistance in colonic HT-29/B6 cell monolayers. Limonene has induced protective and curative effects in the gastrointestinal tract in models of gastric ulcer and colitis, serving as a favorable target to evaluate in these diseases.

4.7 Effects on the respiratory tract

Acute lung injury, when associated with inflammation, is a serious disease that has high rates of morbidity and mortality without any specific drug available for its treatment. Chi *et al.* [80] hypothesized that limonene could prevent acute lung injury induced by LPS intranasal administration in mice. As a result, limonene has been shown to be effective in the protection of the lungs, improving lung function through its anti-inflammatory activity. Limonene was able to decrease the inflammatory infiltration of neutrophils and the activity of myeloperoxidase (MPO). Inflammatory cytokines such as TNF-α, IL-1β and IL-6 are considered therapeutic targets in acute lung injury. Limonene treatment decreased the activity of these cytokines. Some inflammatory mechanisms have been studied and limonene has been able to attenuate the activation of NF-κB, ERK, JNK and p38 MAPK signaling pathways, showing an anti-inflammatory effect on the

prevention of LPS-induced acute lung injury. Bronchial asthma is a chronic inflammatory disease that affects 300 million people worldwide. Individuals who are sensitive to asthma or allergic respiratory diseases are considered to be more sensitive to inhaled irritants because of these airway abnormalities. A study by Hirota *et al.* [81] aimed to assess whether limonene could reduce allergic symptoms and asthma in the model of airway inflammation induced by the dust mite *Dermatophagoides farinae*. The obtained results showed that oral administration of limonene significantly decreases the production of important immunoglobulin markers of allergic inflammation and pro-inflammatory cytokines. In addition, limonene was able to attenuate bronchoconstriction caused by acetylcholine administration in DER mice, showing involvement with cholinergic receptors. These results showed that limonene has a potent therapeutic effect on allergic airways even in the presence of asthma.

The study by Carvalho *et al.* [82-83] demonstrated that the essential oil *Lippia alba*, thanks to its main compounds citral and limonene, induced an antispasmodic (spasmolytic) effect on the tracheal smooth muscle of rats and therefore had a high pharmacological potential for use in respiratory diseases.

4.8 Healing and induction of osteogenesis

According to several studies by Soundharrajan *et al.* [84-85], the authors suggest that limonene could be considered as a promising compound in the development of drugs for bone healing via the induction of osteogenesis. Clinical trials using limonene are not numerous and it would be too early to draw conclusions about its specific effects on human health and disease. However, it must be considered that this can be an advantage point because limonene could offer n'a complementary therapeutic alternative to the research and development of new drugs and treatments.

4.9 Limonene and endocrine system relationship

Limonene has been extensively studied in terms of endocrine disruption. The available data do not reveal endocrine action on the reproductive system as initially suggested by the Structure-Activity Relationships (QSAR) studies. Limonene does not bind to estrogen receptors or androgen receptors [87-88]). As a result, it does not exhibit endocrine disrupting properties [86]. Scientific and regulatory agencies and international agencies have not included it in the lists of substances suspected of causing endocrine disruption. Thus, it is not registered on the "Priority List" of the European Commission (EC-DG Environment) [89], nor on the list of the OSPAR Commission (OSPAR). It is also not listed on the American NGO CPA Clean Production Action (RED) or the US Environmental Protection Agency (US EPA) lists.

5. Conclusion

This review highlights the therapeutic value of natural limonene based on various biological activities as well as its safety (or its potential toxicity). The high availability of limonene in nature, its safety and its wide mechanism of action make this monoterpene a promising preventive agent, and could be also used in a complementary approach to conventional therapeutic drugs using anti-inflammatory and anti-infectious drugs. The considerable amount of scientific data in the literature supports the growing interest in using natural limonene, in its native form or in its natural environment (essential oil), alone or in combination with other drugs, to optimize preventive and therapeutic management of various diseases. Furthermore, a reduced exposure to various synthetic molecules, which have side-effects, could be expected.

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