Review Article

D-Limonene: Safety and Clinical Applications

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Abstract

D-limonene is one of the most common terpenes in nature. It is a major constituent in several citrus oils (orange, lemon, mandarin, lime, and grapefruit). D-limonene is listed in the Code of Federal Regulations as generally recognized as safe (GRAS) for a flavoring agent and can be found in common food items such as fruit juices, soft drinks, baked goods, ice cream, and pudding.

D-limonene is considered to have fairly low toxicity. It has been tested for carcinogenicity in mice and rats. Although initial results showed d-limonene increased the incidence of renal tubular tumors in male rats, female rats and mice in both genders showed no evidence of any tumor. Subsequent studies have determined how these tumors occur and established that d-limonene does not pose a mutagenic, carcinogenic, or nephrotoxic risk to humans. In humans, d-limonene has demonstrated low toxicity after single and repeated dosing for up to one year.

Being an excellent solvent of cholesterol, d-limonene has been used clinically to dissolve cholesterol-containing gallstones. Because of its gastric acid neutralizing effect and its support of normal peristalsis, it has also been used for relief of heartburn. D-limonene has well-established chemopreventive activity against many types of cancers. Evidence from a phase I clinical trial shows a partial response in a patient with breast cancer and stable disease for more than six months in three patients with colorectal cancer. *(Altern Med Rev* 2007;12(3):259-264)

Introduction

D-limonene (1-methyl-4-(1-methylethenyl) cyclohexane) is a monocyclic monoterpene (Figure 1) with a lemon-like odor and is a major constituent in several citrus oils (orange, lemon, mandarin, lime, and grapefruit). Because of its pleasant citrus fragrance, d-limonene is widely used as a flavor and fragrance additive in perfumes, soaps, foods, chewing gum, and beverages.[1] D-limonene is listed in the Code of Federal Regulation as generally recognized as safe (GRAS) for a flavoring agent.[2] The typical concentration of d-limonene in orange juice, ice cream, candy, and chewing gum is 100 ppm, 68 ppm, 49 ppm, and 2,300 ppm, respectively.[1]

Dietary intake of d-limonene varies depending on the types of foods consumed. Daily U.S. per capita consumption of d-limonene from both its natural occurrence in food and as a flavor is estimated to be 0.27 mg/kg body weight/day for a 60 kg individual (0.27 mg/kg body weight x 60 kg=16.2 mg/person/day).[3] It has been reported that in an Arizona population, the daily d-limonene intakes from citrus juice and peel are 20-40 mg/day and 50-90 mg/day, respectively.[4]

Absorption, Distribution, and Metabolism

Oral administration of d-limonene is rapidly and almost completely absorbed in the gastrointestinal tract in humans as well as animals.[5-8] In humans, ingestion of 1.6 g (14C)d-limonene resulted in an excretion of 52-83 percent of the dose in the urine within 48 hours.[6]

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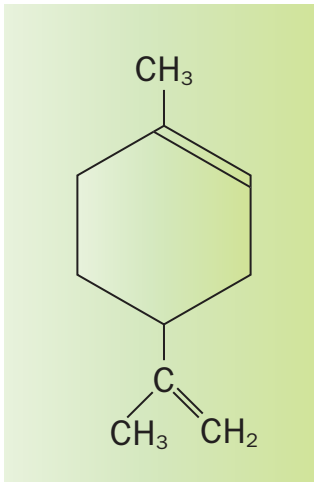
D-limonene is rapidly distributed to different tissues in the body and is readily metabolized. D-limonene and/or its metabolites are detectable in serum, liver, lung, kidney, and many other tissues,[5] with higher concentrations detected in adipose tissue and mammary gland than in less fatty tissues.[7] The half-life of d-limonene in humans has been estimated to be 12-24 hours,[7] and excretion occurs primarily through the urine.[5,6] No accumulation of the metabolites was found after repetitive dosing for 21 days.[8]

D-limonene is metabolized to oxygenated metabolites in rats and humans. In humans, the predominant circulating metabolites are perillic acid, dihydroperillic acid, and limonene-1-,2-diol. Other metabolites in plasma include limonene-8,9-diol and perillic acid isomer.[8-10]

Figure 1. D-Limonene



One human study observing healthy individuals drinking 30-40 ounces of lemonade containing 447-596 mg d-limonene found plasma concentrations of perillic acid peaked one hour after lemonade consumption and rapidly declined with time. Maximum plasma concentration of perillic acid was 2.08-13.98 μM and were undetectable after 24 hours of lemonade consumption.[11]

Urinary metabolites include glucuronides of the two isomers of perillic acid, dihydroperillic acid, limonene-8,9-diol, and monohydroxylated limonene.[8,10] About 25-30 percent of an oral dose of d-limonene in humans was found in urine as d-limonene-8,9-diol and its glucuronide; about 7-11 percent was eliminated as perillic acid and its metabolites.[6]

Safety

D-limonene is considered to have fairly low toxicity. It has been tested for carcinogenicity in mice and rats. Although initial results showed d-limonene increased the incidence of renal tubular tumors in male rats, female rats and mice of both genders showed no evidence of any tumor. Subsequent studies have determined how these tumors occur and established that d-limonene does not pose a mutagenic, carcinogenic, or nephrotoxic risk to humans. In humans, d-limonene has demonstrated low toxicity after single and repeated dosing for up to one year.

Acute and Sub-acute Toxicity Study

The oral LD50 for d-limonene in male and female mice is reported to be 5.6 and 6.6 g/kg body weight, respectively, while LD50 in male and female rats is reported to be 4.4 and 5.1 g/kg body weight, respectively.[12]

No histological abnormality was found 30 minutes after infusion of 10 mL d-limonene into the duodenum of rats.[13] In pigs, 20 mL d-limonene was infused into the gallbladder once daily for two days. Twenty-four hours after the last infusion, histological examination found no abnormality in the mucosa of the gallbladder, common bile duct, or duodenum, which were directly in contacted with d-limonene.[14] In dogs, 10 mL d-limonene was infused daily for seven days via a cholecystostomy tube. On the day following the last infusion, no major abnormality was found except slight inflammatory cell infiltration and fibrosis in the duodenal papilla.[13]

In 1990, the National Toxicology Program (NTP) investigated the toxicity of d-limonene (>99% pure) at doses ranging from 413-6,600 mg/kg daily administered to rats and mice five days/week for three weeks. No signs of compound-related toxicity were noted at doses <1,650 mg/kg daily.[15,16]

Another study observed decreased weight gain and even death in male rats starting at a dose of 600 mg/kg daily. As doses reached 1,200-2,400 mg/kg/day, surviving male rats developed rough hair coats, lethargy, and excessive lacrimation. Nephropathy was noted in all male rats at the end of the study. In the case of mice, decreased body weight gain, lethargy, and rough hair coats were observed in male mice given the two highest doses of d-limonene (1,000 and 2,000 mg/kg daily). No other compound-related signs of toxicity or lesions were noted.[12,15,16]

Chronic Toxicity Studies

In a two-year study, female rats given 600 mg/kg daily experienced significantly lower survival compared to controls.[15] In male rats, microscopic evidence of compound-related nephropathy was noted.[15] D-limonene belongs to a group of hydrocarbons shown to induce a unique nephropathy syndrome in rats after subacute or chronic exposure. The nephropathy is associated with α_{2u}-globulin (α_{2u}-g) accumulation in hyaline droplets, not an appropriate endpoint for humans because no such reaction occurs in humans.[16]

After week 28 of the study, female mice exposed to 1,000 mg/kg d-limonene daily decreased in mean body weight by 5-15 percent compared to their respective vehicle controls.[15] Increased incidence of multinucleated hepatocytes and cytomegaly was observed in male but not female mice. As a result of the presence of liver lesions, a "No Observed Adverse Event Level" (NOAEL) dosage of 250 mg/kg/day and a "Lowest Observed Adverse Event Level" (LOAEL) dosage of 500 mg/kg/day was calculated.[15]

Mutagenicity and Carcinogenicity Study

D-limonene elicited no mutagenicity in four strains of *S. typhimurium* (TA98, TA100, TA1535, or TA1537). Furthermore, no induction of chromosomal aberrations or sister chromatid exchange in cultured Chinese hamster ovary cells was observed.[15] Additionally, d-limonene did not contribute to cell mutations in the liver or kidney of rats.[17]

Although male rats experienced an increased incidence of tubular cell hyperplasia, adenomas, and adenocarcinomas of the kidney, no evidence of carcinogenic activity was observed in female rats or male or female mice.[15]

Human Safety Studies

In an early study, five healthy males received a single dose of 20 g d-limonene. Although subjects complained about increased bowel movements (2-3 times daily) and tenesmus, blood tests showed no abnormalities in liver (total protein, bilirubin, cholesterol, AST, ALT, and alkaline phosphatase), kidney (BUN), or pancreatic (amylase) functions.[13]

D-limonene has also been found to be safe, without gradable toxicity, when 100 mg/kg (equivalent to about 7 g for an average adult male) was ingested. Only mild eructation for 1-4 hours post-ingestion, mild satiety for 10 hours post-ingestion, and slight fatigue for four hours post-ingestion were reported.[9]

In a dose escalation study of 32 patients with refractory solid tumors, d-limonene was given orally at 0.5-12 g/m^2/day (1-24 g/day, considering an average area per person is 1.9 m^2). Patients initially received d-limonene for 21 days. The maximum tolerated oral dose was 8 g/m^2/day (15 g/day). Nausea, vomiting, and diarrhea were the only side effects observed and were dose dependent. One breast cancer patient was on the dose of 8 g/m^2/day (15 g/day) for 11 months. The authors concluded that d-limonene had low toxicity after single and repeated dosing for up to one year.[8]

Nephropathy seen in rats after high-dose limonene does not appear to be possible in humans, since neither the quantity nor type of protein that binds d-limonene or d-limonene-1,2-oxide is present. The protein content of human urine is very different from rat urine, as humans excrete very little protein if any (1 percent or less of the concentration found in urine of male rats). There is also no protein in human plasma or urine identical to α_{2u}-globulin and no α_{2u}-g-like protein has been detected in human kidney tissue. Although d-limonene-1,2-oxide binds to α_{2u}-g, no other proteins, particularly those synthesized by humans, bind d-limonene-1,2-oxide. Finally, there is no evidence that any human protein can contribute to a renal syndrome similar to α_{2u}-globulin nephropathy.[12,16]

Clinical Applications

Because it is a solvent of cholesterol, d-limonene has been clinically used to dissolve cholesterol-containing gallstones. It has also been used to relieve heartburn, because of its potential for gastric acid neutralization and its support for healthy peristalsis. D-limonene has well-established chemopreventive activity against many types of cancers. Evidence from a phase I clinical trial shows a partial response in a patient with breast cancer and stable disease for more than six months in three patients with colorectal cancer. (Table 1)

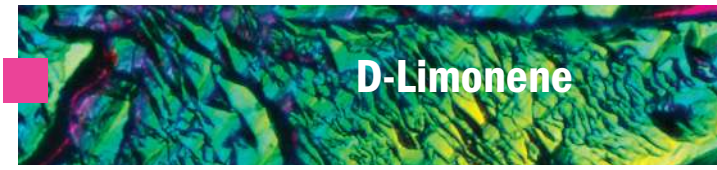


Table 1. Clinical Applications of D-Limonene

Indication	Evidence
Gallstone Dissolution	Uncontrolled clinical trials; animal studies; *in vitro* lab findings
Heartburn Relief/GERD	Controlled clinical trial; uncontrolled clinical trial; *in vitro* lab findings
Anticancer Activity	Uncontrolled clinical trials; case reports; animal studies; *in vitro* lab findings

Gallstone Dissolution

In vitro, d-limonene dissolved human gallstones within two hours.[14] In animals, infusion of d-limonene into the gallbladder dissolved and disintegrated gallstones, which were excreted through the common bile duct.[14] In patients post gallstone surgery, infusion of 20 mL d-limonene every other day dissolved gallstones overlooked during surgery. In some patients gallstone dissolution occurred after only three infusions.[14]

A study with 200 patients reported a direct infusion of 20-30 mL d-limonene (97% solution) completely or partially dissolved gallstones in 141 patients. Stones completely dissolved in 96 cases (48%); partial dissolution was observed in 29 cases (14.5%); and in 16 cases (8%) complete dissolution was achieved with the inclusion of hexamethaphosphate (HMP), a chelating agent that can dissolve bilirubin calcium stones. All the stones were between 0.5 and 1.5 cm with an average diameter of 1.0 cm. The duration of the treatment ranged from three weeks to four months.[18]

Gastroesophageal Reflux

D-limonene has been shown to be effective in relieving occasional heartburn and gastroesophageal reflux disorder (GERD). In a clinical setting, 19 adults suffering from chronic heartburn or GERD were invited to use d-limonene to relieve their symptoms. All participants had a history of chronic heartburn or GERD, with symptoms ranging from mild/moderate to severe for at least five years. Before taking d-limonene, each participant was asked to rate the frequency and severity of symptoms on a scale of 1-10, with 1 corresponding to complete relief and 10 corresponding to severe and/or painful symptoms that occur every day. Most participants had an initial severity and frequency rating of 5 or greater. Participants were asked to discontinue current treatments (OTC and/or prescription medications), take one capsule containing 1,000 mg d-limonene every day or every other day, and rate symptoms daily using the frequency/severity index described above. On the second day of taking d-limonene, 32 percent of participants experienced a significant relief of symptoms (severity rating=1-2); this relief rate improved gradually during the regimen. By day 14, 89 percent of participants achieved complete relief of symptoms.[19]

In a double-blind, placebo-controlled study, 13 participants suffering from mild/moderate to severe heartburn/GERD were randomized to d-limonene or placebo. Seven participants in the d-limonene group received 1,000 mg d-limonene once daily or every other day, while six participants received an identical capsule containing soybean oil (placebo). Each participant was asked to rate the frequency and severity of symptoms on a scale of 1-10 described above. On day four, 29 percent of participants in the d-limonene group experienced significant relief of symptoms (severity rating=1-2), compared to no relief of symptoms in the placebo group. By day 14, 86 percent of participants achieved complete relief of symptoms, compared to 29 percent of participants in the placebo group.[19]

Results from these two studies suggest the beneficial effects of d-limonene appear to develop over time, with the best results attained after following a 10-capsule regimen. The mechanisms of actions of d-limonene have not been fully elucidated. *In vitro* study suggests it may neutralize the effect of gastric acid by coating the stomach wall and protecting the mucosal lining from gastric acid exposure.[19] Some researchers

believe d-limonene may support healthy peristalsis. In a study with guinea pig ileum and rat vas deferens, d-limonene increased the resting tone of these tissues.[20]

Anticancer Activity

Animal studies have set the stage for further investigation into the chemoprotective activity of d-limonene for several types of cancer. Several experiments demonstrated inhibition of chemically-induced mammary cancer in rodents administered either orange peel oil or pure d-limonene.[21-24] Inhibition occurs in either the initiation or promotion phases, depending on the chemically-induced medium used.[25-27] Other animal trials demonstrated d-limonene inhibited development of liver cancer, pulmonary adenoma, and forestomach tumors.[28-30]

D-limonene induces phase I and phase II carcinogen-metabolizing enzymes (cytochrome p450), which metabolize carcinogens to less toxic forms and prevent the interaction of chemical carcinogens with DNA. D-limonene has been shown to enhance gastrointestinal UDP-glucuronosyltransferase (UGT) activity in rats.[31] It also inhibits tumor cell proliferation, acceleration of the rate of tumor cell death and/or induction of tumor cell differentiation. Furthermore, d-limonene inhibits protein isoprenylation. Many prenylated proteins regulate cell growth and/or transformation. Impairment of prenylation of one or more of these proteins might account for the antitumor activity of d-limonene.[25] It was found that d-limonene attenuates gastric cancer through increasing apoptosis, while decreasing DNA synthesis and ornithine decarboxylase activity of cancer cells.[26,27] D-limonene inhibits hepatocarcinogenesis via inhibition of cell proliferation, enhancement of apoptosis, and blockage of oncogene expression.[32,33]

D-limonene may also exhibit immune-modulating properties. One animal study observed increased survival in lymphoma-bearing mice placed on a high d-limonene diet. These mice also demonstrated increased phagocytosis, microbicidal activity, and nitric oxide production.[34]

In a phase I pharmacokinetics study of d-limonene in patients with advanced cancer, a female breast cancer patient demonstrated partial beneficial response to d-limonene at a dose of 8 g/m^2/day. Axillary and supraclavicular lymph nodes containing metastatic infiltrating ductal carcinoma remained stable during the first five treatment cycles. At the beginning of the sixth cycle, supraclavicular lymphadenopathy was reduced by >50 percent, and by the 14th course axillary lymph nodes were no longer palpable; bone pain decreased as well. Response was maintained for 11 months before progression of cancer in the bone forced the patient to withdraw from the study.

Three individuals with colorectal carcinoma, while on d-limonene, were able to suspend progression of the disease for over six months. Similarly, d-limonene at a dosage of 0.5 g/m^2/day was able to halt progression of cancer for nine months in a patient diagnosed with locally advanced mucinous cystadenocarcinoma of the appendix. A patient with presacral recurrence of an adenocarcinoma in the sigmoid colon experienced a minor reduction (<50%) in tumor size at a dose of 0.5 g/m^2/day for 12 months. Another patient with local retrovesical recurrence of colorectal adenocarcinoma remained stabilized on 1 g/m^2/day (2 g/day) for 7.5 months.[8]

One epidemiological study reported that people without epithelial cell carcinomas consumed significantly more citrus peel, rich in d-limonene, than those having epithelial cell carcinomas. Moreover, a dose-response relationship was observed between higher citrus peel in the diet and reduced risk of skin cell carcinoma. The authors concluded that citrus peel consumption, the major source of dietary d-limonene, might have a potential preventive effect on squamous cell carcinoma.[4]

While these case and epidemiological reports are of interest, larger, more comprehensive studies are necessary to confirm d-limonene's effectiveness as a potential chemopreventive and treatment agent.

Conclusion

D-limonene is considered to be a chemical with fairly low toxicity. Studies have determined d-limonene does not pose a mutagenic, carcinogenic, or nephrotoxic risk to humans.

D-limonene has been clinically used to dissolve cholesterol-containing gallstones. It has also been used for relief of heartburn/GERD, because of its gastric acid neutralizing effect and improvement of peristalsis.

D-limonene has well-established chemopreventive activity against many types of cancers. Evidence from a phase I clinical trial shows a partial response in a patient with breast cancer and stable disease for more than six months in three patients with colorectal cancer.

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