Food Sci. Biotechnol. Vol. 18, No. 4, pp. 833 ~ 841 (2009)

RESEARCH REVIEW

Food Science and Biotechnology

Limonene and Its Oxyfunctionalized Compounds: Biotransformation by Microorganisms and Their Role as Functional Bioactive Compounds

Mário Roberto Maróstica Junior* and Gláucia Maria Pastore[1]

Department of Food and Nutrition, Faculty of Food Engineering, State University of Campinas, 13035-388, Campinas, Brasil
[1]Department of Food Science, Faculty of Food Engineering, State University of Campinas, 13035-388, Campinas, Brasil

Abstract Monoterpenes, in special limonene and its derivatives, are well studied in the literature due to their several properties. They are well recognized as major components of essential oils; some of them, are important industry residues, and others present some important biological activities. In this review, the biotransformation of the inexpensive limonene into flavor compounds was briefly reviewed and the main pathways for limonene biotransformation are presented. Furthermore, some important biological properties of these compounds were also considered, like bactericidal activity, induction of immune response, and role in disease prevention, with a little emphasis on some possibilities related to the mechanisms of anticancer action.

Keywords: limonene, limonene derivatives, biotransformation, biological property, anticancer activity

1. Introduction

Limonene is a monoterpene found in abundance in nature. It is spread mainly in trees, herbs, and fruits. *R*-(+)-Limonene is well known as the major component of orange peel oil. In orange juice industries, the peel oil is recovered after concentration steps. Limonene possesses generally recognized as safe (GRAS) status and has several applications in industry: the orange peel oil can be used in solvents to paints, in household products, in herbicides, in pipeline cleaners, in aroma ingredients, and other products (1).

Limonene's molecule is very similar to the ones from very impacting aroma compounds, like carvone, α-terpineol, mentol, etc. This, together with the increasing amounts of limonene available from orange juice processing (specially in countries like Brazil, which produces high amounts of concentrated orange juice), increased the searches on the conversion of the inexpensive *R*-(+)-limonene in oxyfunctionalized compounds (2).

The main advantages of biocatalytic processes are related to the specificity of the reaction and, generally, allow the production of regio and stereoselective products. These compounds can be labeled as natural according to the legislation of USA and countries from Europe. On the other hand, low yields can be generally regarded as the major problem on the bioconversion/biotransformation of limonene (3).

The first report on the biocatalytic conversion of limonene was on the 1960s. Since then, several investigations related microorganisms, plant cells, enzymes, and microalgae capable of transforming limonene in many oxyfunctionalized derivatives through specific pathways (4,5).

*Corresponding author: Tel: +55-19-3521-4078; Fax: +55-19-3521-4059
E-mail: mario@fea.unicamp.br
Received January 16, 2009; Revised February 24, 2009;
Accepted March 14, 2009

In order to counteract the low yields, several techniques have arisen. Some of them are: suspended or immobilized cells, induction of enzymes through adaptation of precultures with substrates and/or products, two phase reaction systems, and other techniques (6).

Some other reviews about biotransformation of limonene can be also found: Braddock and Cadwallader (7), Duetz *et al*. (2), and Marostica-Jr and Pastore (8).

On another way, some monoterpenes are described also as bioactive compounds. Some reports describe the effects of limonene and some of its oxyfunctionalized derivatives on health promotion. Limonene is one of the most studied monoterpenes related to biological effects and several investigations relate the action of perillyl alcohol as a possible anticancer compound (9). Recent investigations revealed that the monoterpene perillyl alcohol has preventive and therapeutic effects in a wide variety of preclinical tumor models. Several mechanisms of cancer prevention were proposed for limonene and perillyl alcohol and will be briefly discussed on this review.

This review focused mainly on two points: 1) the biotransformation of limonene to produce 'natural' flavor compounds; and 2) bioactivity of limonene and its oxyfunctionalized compounds. The Fig. 1 shows the structures of limonene and some of its main oxyfunctionalyzed derivatives obtained by biotransformation processes.

2. Biotransformation of Limonene in Aroma Compounds by Microorganisms

2.1. Main pathways in the biotransformation of limonene into aroma compounds

There are several reports about the oxyfunctionalized products derived from limonene degradation. There are also recent publications about the main pathways for limonene oxidation. Van der Werf *et al.* (10) reviewed the most important reactions that take place in limonene to

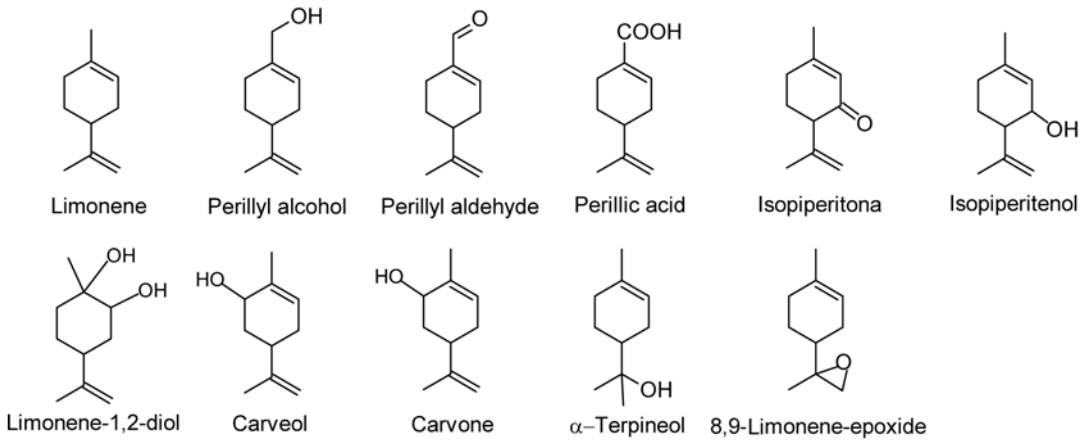

Fig. 1. Structures of limonene and its main oxyfunctionalyzed derivatives.

generate oxyfunctionalized compounds. The authors described 5 main possibilities for oxidation of the inexpensive limonene:

1) Oxidation of C7 to produce perillyl compounds
2) Oxidation of C1-C2 double bound to generate the respective diol
3) Oxidation of C6 to produce carveols and carvone
4) Oxidation of C8-C9 double bond to produce α-terpineol
5) Oxidation of C3 to produce isopiperitenol and isopiperitone

One year after, the same research group reported another pathway (11) for the oxidation of limonene by a bacterial strain:

6) Oxidation of C8-C9 double bond to produce 8,9-limonene epoxide

Furthermore, the website of Minessota University shows a very complete diagram with the main pathways involved on limonene transformation (12).

2.2. Brief description of some of the catalysts involved on each of the pathways described

Below there is a brief description of the main catalysts able to transform limonene according to each of the 6 pathways described above. Although far from providing a complete list of the catalysts reported in the literature, this section relates some of the investigations on the literature related to the products of biotransformation reported. The information related here comes from scientific papers and do not include patents.

2.2.1. Biocatalysts able to perform the oxidation on C7 to produce perillyl compounds Pseudomonas strains seem to be the most important ones on the biotransformation of limonene into perillyl compounds. A Pseudomonas strain was reported as the first catalyst able to convert limonene into perillyl derivative (4). Perillic acid was recovered from the medium that contained limonene as the sole carbon source. After that, several bacteria strains were reported as capable of biotransform limonene. Another investigation reported the ability of different Pseudomonas specie (*P. gladioli*) to produce perillic acid from limonene. Another report using also a *P. putida* leads to a final concentration of 3 g/L perillic acid (13). Glycerol was used as co-substrate to strain growth and was necessary to the production of the stable final product perillic acid. Perillyl alcohol, known by its biological properties, was also obtained by *Pseudomonas* species (*P. putida*) according to the work reported by Chatterjee and Bhattachyya (14). The bacterial strain was found to be resistant to a concentration of 0.2% of limonene, whilst 0.5% of substrate leads to inhibition of strain development.

The production of perillyl derivatives is also described to filamentous fungi. A strain of *Aspergillus cellulosae* (15) and another of *Fusarium oxysporum* (6) described recently are among the strains that can produce perillyl alcohol. Furthermore, the hydroxylation of limonene C7, leading to perillyl alcohol in a psychrotrophic fungi, *Mortierella minutíssima*, isolated from artic soil, showed to be improved at 15°C in comparison to higher temperatures (16).

A more recent investigation reported the use of solid phase microextraction (SPME) to screen microorganisms able to biotransform limonene (17). More than 40 cultures were evaluated according to the biotransformation of orange oil with 94% of limonene (17). The strain *Penicillium* sp. 2360 showed to transform limonene into perillyl alcohol, and this result was confirmed by submerged liquid cultures experiments. Unfortunately, the reaction resulted in low yields of products.

2.2.2. Biocatalysts able to perform oxidation on C1-C2 double bound to generate the respective diol Limonene-1,2-diol is a biotransformation product obtained from the biotransformation of limonene by yeasts and filamentous fungi. Noma *et al.* (15) reported that limonene-1,2-diol produced by the action of *A. celullosae* on limonene. However, some years before, one of the most interesting studies on biotransformation of limonene on oxyfunctioalized products was performed by the filamentous fungi *Corynespora cassiicola* DSM 62475 (18). The products formed were *cis*- and *trans*-limonene-1,2-diol, with final concentrations of 0.2 and 1.5 g/L, respectively. The scaled-up biotransformation was carried on a 70-L bioreactor, and after 96 hr, they were able to recover 900 g of the biotransformation products. A more recent investigation reported the production of limonene-1,2-diol by a *C. cassiicola* strain (19). The authors reported a successful method of selecting microorganisms that can biotransform

limonene into flavor compounds using SPME technique.

A bacterial strain, *Rhodococcus erythropolis*, also proved to biotransform limonene (the terpene was the sole C-source) into limonene-1,2-diol (10). The authors reported the 2 steps biotransformation of limonene. On the first step, the hydrocarbon monoterpene is transformed into limonene-1,2-epoxide and the reaction is mediated by a limonene-1,2-monoxigenase. On the second step, the epoxide is converted into limonene-1,2-diol with a limonene-1,2-epoxide-hidrolase, which is described as a very active and inductive enzyme. Both enantiomers were degraded in similar ways, but the stereochemical configurations of *R*-(+)- and *S*-(-)-limonene are opposite. This was the first report about microorganisms that can transform limonene through a pathway started by the 1-2 double bonds.

2.2.3. Biocatalysts able to perform the oxidation on the C6 to produce carveols and carvone Carveols and carvone are very odor-active substances and are well know by the characteristic mint aroma. The pioneer Indian group reported the biological oxidation of limonene-C6 for the first time (4,5). Since than, several microorganisms were studied about their ability in transforming limonene into carvone.

In 1999, a German group reported the regioselective production of carveol and carvone by the basidiomycete *Pleurotus sapidus* (20). When the substrate was added in a gaseous form, the final yields were 70 and 30 mg/L for carveol and carvone, respectively. *Penicillium digitatum* and *Penicillium italicum* isolated from orange peel also showed to be able to attack limonene in C6-position (21). The initial substrate concentration was 0.5%. The authors reported that higher concentrations diminished the microorganism's growth. The other products else than caveol and carvone resulted from the biotransformation, what indicated that the system was not regiospecific. The other compounds formed were: *cis* and *trans*-*p*-mentha-2,8-dien-1-ol, *p*-menta-1,8-dien-1-ol, and *p*-menta-8-eno-1,2-diol.

Up to now, the plant enzymes showed greater regiospecificity; however, unfortunately, the yields are far from being great enough for a direct industrial scale application. A limonene-6-hydroxylase from *Mentha* spp. showed great specificity in converting limonene in carveol (22). Whole cells of *Solanum aviculare* and *Dioscorea deltoidea* were able to convert *R*-(+)-limonene in racemic mixtures of carvone and carveol (23). However, cells of *Rhodococcus opacus* PWD4 transformed limonene exclusively on position 6, generating (+)-*trans*-carveol as the unique biotransformation product with about 95% of conversion (24).

2.2.4. Biocatalysts able to perform the oxidation on C8-C9 double bond to produce α-terpineol α-Terpineol can be produced by limonene oxidation mediated by a great number of microorganisms. Kraidman *et al.* (25) reported for the first time the biotransformation of limonene in α-terpineol by a strain of *Cladosporium* sp. A series of 3 papers reported the production and several techniques to improve the yields of α-terpineol biotransformed by a *P. digitatum* strain (26-28). The authors confirmed that a increasing of 12 times on activity resulted from sequential addition of the substrate limonene. Some of the co-solvents tested proved to double the activity in some cases. Laboratory scale biotransformation experiments lead to final yields of 3.2 g/L.

Another investigation reported the isolation of a *P. digitatum* from mandarin using SPME (19). Two years after, the same research group reported that the strain was able to regiospecifically convert *R*-(+)-limonene into *R*-(+)-α-terpineol with an yield of 93% using ethanol as co-solvent (29) in submerged liquid culture experiments. The strain was able to biotransform *S*-(-)-limonene also, but only trace amounts of biotransformation products were detected.

The use of residues on biocatalytic processes is an interesting topic and has arisen as a good alternative to minimize production costs. A recent investigation reported the biotransformation of limonene using 2 industrial residues: limonene source was obtained from a citrus juice industry and the culture medium was manipueira, a waste from manioc flour processing (6). A *F. oxysporum* strain isolated from a Brazilian tropical fruit was able to grow well on manipueria medium, as manioc medium is a very rich source of carbohydrates and minerals. The growth mycelium was transferred into a mineral medium and the orange oil (more than 94% of limonene) was added as the sole C-source for biotransformation procedures. The strain biotransformed limonene into α-terpineol as main product. Final yields were not high enough for industry application. The same authors proved that another terpene, citronellol, can be converted into oxidation products by a *Penicillium* sp. strain using a very similar proceeding (30).

Faced on the great results obtained with the excellent filamentous fungi strain, the same research group performed the optimization of the culture conditions in order to increase the yields of α-terpineol production (31). A Plackett-Burman screening design was used to study the effects of the medium composition (glucose, peptone, yeast extract, malt extract, and pH), the presence of a co-substrate (a biosurfactant obtained from synthesis from a manipueira medium by the action of a *Bacillus subtilis* strain), the cultivation conditions (temperature, agitation), the substrate concentration and the inoculum/culture medium ratio. After that, an optimization was obtained by using a response surface methodology. The best condition was 0.5%(v/m) *R*-(+)-limonene in pure distilled water as the culture medium with an inoculum/culture medium ratio of 0.25 (m/m) and 72 hr cultivation at 26ºC and 240 rpm. The final concentration of *R*-(+)-α-terpineol was 2.4 g/L (6 times greater than the initial conditions). The authors reported that the presence of a biosurfactant from *B. subtilis* did not significantly increase the yield.

2.2.5. Biocatalysts able to perform the oxidation on C3 to produce isopiperitenol and isopiperitone There are few reports about the biotransformation of limonene in isopiperitenol and isopiperitone. In the first one, the authors reported the biotransformation of *R*-(+) and *S*-(-)-limonene by an *A. celulosae* strain (15); (+) and (-)-isopiperitone were obtained as the main metabolites, however, other minor products were also present. After that, a yeast identified as *Hormonema* sp. was able to convert *R*-(+)-limonene in *trans*-isopiperitenol. The final

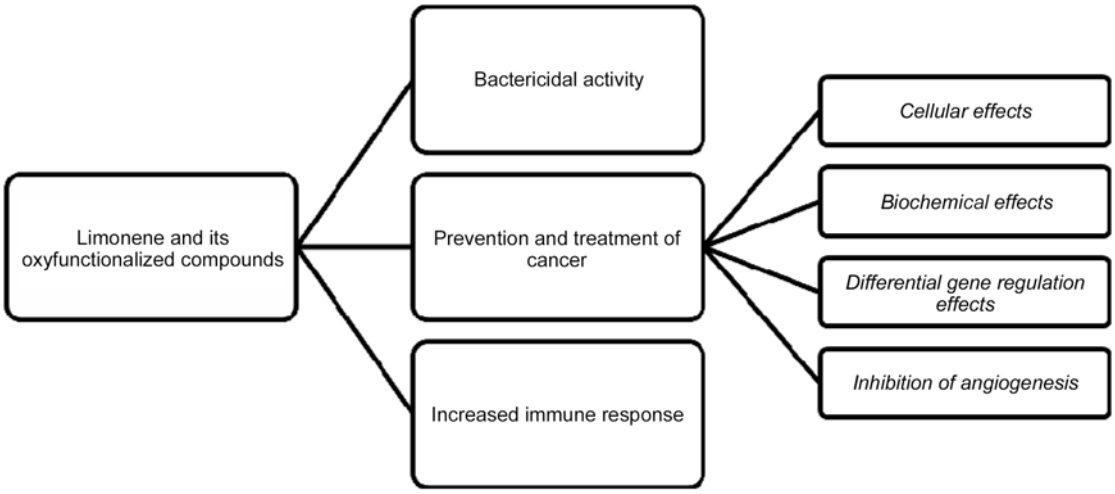

Fig. 2. Some of the bioactive roles of limonene and its oxyfunctionalyzed derivatives.

yield was 0.5 g/L after 12 hr incubation. Unfortunately, the authors reported little reproducibility due to morphological mutations examined by microscope.

(-)-Isopiperitenol was an intermediate compound from the biotransformation of *S*-(-)-limonene in (-)-mentol by *Mentha* ssp. (22).

2.2.6. Biocatalysts able to perform the oxidation on C8-C9 double bond to produce 8,9-limonene epoxide The first report of epoxidation of limonene to form limonene-8,9-epoxide was done by van der Werf *et al*. (11). The authors reported the isolation of a strain of *Xanthobacter* sp. able to grow on cyclohexane as the sole C-source. When the strain was exposed to limonene, the unique product detectable after the conversion was limonene-8,9-epoxide. *R*-(+)- and *S*-(-)-limonene were 100% converted in (*4R,8R*)-limonene-8,9-epoxide and in 78:22 mixture of (*4S,8R*)-limonene-8,9-epoxide: (*4S,8S*)-limonene-8,9-epoxide, respectively. The authors also reported that the inhibition caused by the biotransformation products could not be minimized with the addition of co-solvents.

3. Biological Activities of Limonene and Its Oxy-functionalized Compounds

Figure 2 summarizes some of the main biological effects of limonene and its derivatives.

3.1. Bactericidal activity of essential oils

Essential oils have been considered as active compounds against the growth of several microorganisms. Essential oils have showed bactericidal and fungicidal activities. Essential oils are mainly composed by terpenes, terpenoids, and aromatic compounds. One essential oil can contain from 20 to 60 different compounds. Because of this large number of components, essential oils seem not to have a specific way of action (32).

In general, all the compounds present in an essential oil are lipophilic; which confer them the potential of entering inside some cells and cell structures through the disruption and solubilization of some of their membrane components (phospholipids, lipids as well as polysaccharides). This lead to a higher membrane permeabilization and loss of ions; what results in a reduction of membrane potential, decrease of the proton pump and depletion of the ATP pool (33). The effects of essential oil constituents achieve other cell structures other than plasmatic membrane: cytoplasm and nucleus are also altered (34). The membrane of other organelles, such as mitochondria and peroxisomes are also modified. These effects on cell structures are some of the more accepted explanations for their well known cytotoxic, including bactericidal and fungicidal, effects.

Essential oils are normally obtained by steam or hydro-distillation. However, orange essence oil is obtained through a different process in industry. The orange essence oil is normally obtained from the processing of orange juice, more particularly, during concentration; and the oil is separated from aqueous portion by centrifugation (34). The unique flavor of orange juice interested researchers from the entire world, which makes orange oil one of the most studied so far (36). Several compounds contribute to the flavor of orange essence oil, but the terpenes are the major ones. Limonene is the major compound (more than 94%) present in orange essence oil (6).

Limonene is a well-known cytotoxic compound against several bacteria, such as *Staphylococcus epidermidis, Escherichia coli, Pseudomonas aeruginosa,* and *Klebsiella pneumonia* (37). The bactericidal and bacteriostatic activities of limonene were recently assessed by a French group (38). The authors studied the effects of several terpenes on bactericidal and bacteriostatic activities against *Lysteria monocytogenes*. The authors found values of 0.047 and 0.028% for minimal inhibitory concentrations to *L. monocytogenes* for *R*-(+)- and *S*-(-)-limonene, respectively. Furthermore, the weakest bactericidal concentrations for *R*-(+)- and *S*-(-)-limonene were 0.208 and 0.150%, for the same bacteria strain tested.

As commented previously in this section, the more accepted mechanisms for bactericidal and bacteriostatic activities of terpenes and, in special, of limonene, are attributed to their low solubility in water. One of the tools for water solubility measure is the log P, the decadic logarithm of the partition coefficient between *n*-octanol and water, which is expresses the toxicity of water-

immiscible organic solvents towards microorganisms (39). Log P between 1 and 5 is considered strong cytotoxicity. Limonene (log P=4.8) cause toxicity to microorganisms leading to membrane fluidity and inactivation of energy metabolism (20).

3.2. Role of monoterpenes on the prevention and treatment of some diseases

3.2.1. The role of monoterpenes on immune system Regulation of immune responses is a topic of interest in immunology research. Some investigations were already focused on the study of the action of several foods and nutrients on stimulation of immune response, such as probiotics, selenium, and some vitamins (A, C, and E) (40). Furthermore, some investigations focused on the possible immuno-modulatory effects of plants (immuno-modulators can modify the body's defense mechanism to control immune response) (41).

Unfortunately, few reports have been done on the possible effects of monoterpenes on the immune system, including in cell and animal models. One of these investigations studied the action of 3 monoterpenes in immune system of mice. The results showed that the monoterpenes studied were able to stimulate the immune system of the animals. Administration of terpenoids increased the total antibody production, antibody producing cells in spleen, bone marrow cellularity and α-esterase positive cells significantly compared to the normal animals. Maximum concentrations of white blood cells were achieved on the 12th day of treatment for carvone and on the 9th day for limonene and perillic acid (41).

In a previous investigation, Evans *et al*. (42) evaluated the effect of limonene on lymphocyte proliferation and antibody responses. They reported that *R*-(+)-limonene concentration and administration time influenced lymphocyte proliferation and specific antibody responses in normal BALB/c mice. In another investigation, Hamada *et al*. (43) investigated the relationship between the absorptive pathway and the immune responses of the lung. The phagocytic function of alveolar macrophages after oral administration of *R*-(+)-limonene in rats was assessed. The authors demonstrated that *R*-(+)-limonene increases alveolar macrophage activation in normal rats; furthermore, the activity of alveolar *in vitro* incubation with *R*-(+)-limonene increased in a dose-dependent manner.

Another study investigated the survival of lymphoma-bearing mice fed with a diet with *R*-(+)-limonene (44). The authors used 2,4-dinitrofluorobenzene (DNFB) to sensitize the mice and the T-cells subpopulations were assessed by flow cytometry. The role of limonene and its metabolites, like perillyl alcohol and perillic acid, was determined by lymphocyte proliferation and macrophage nitric oxide (NO) production. The authors concluded that *R*-(+)-limonene can modulate the immune response, and clinical applications could be developed.

3.2.2. The role of monoterpenes on the prevention and treatment of cancer Several investigations pointed out that some monoterpenes can be useful in the prevention and treatment of some cancers. Undoubtedly, limonene and perillyl alcohol are the most studied monoterpenes that can show efficacy on chemotherapeutic properties in rat and human cancer models. Breast, liver, lung, and pancreatic cancers are some of the well-known ones that can be inhibited by the above cited monoterpenes (9). Monoterpenes are effective in treating early and advancer cancers and they are effective against neuroblastomas and leukemias (45).

These compounds inhibit both initiation and promotion in animal models of tumorigenesis and can induce regression of established rodent mammary and pancreatic tumors (46). During the initiation phase of carcinogenesis, the more accepted mechanism is the prevention of the interaction of carcinogens with DNA. During the promotion phase, they can act on the inhibition of cancer cell development and migration (47).

Some researches revealed that the tumor regression capacity of perillyl alcohol is related to the production of its metabolites, perillic and dihydroperillic acids (9). Samaila *et al.* (48) evaluated the effects of perillyl alcohol and perillic acid (alone or in combination with radiation) on human head and neck cell carcinoma cell lines. They found that the monoterpenes significantly inhibited growth of the cellular lines studied.

A Brazilian group has reported its recent advances in the treatment of brain tumors with perillyl alcohol (49-51). The authors used intranasal delivery in human patients as it allows some agents to enter blood-brain barrier and enter the central nervous system, reducing side effects. The authors described success in the treatments as the significant reduction in the tumors.

3.3. Possible mechanisms of monoterpene on cancer prevention

The possible mechanisms of action of monoterpenes on cancer prevention and treatment process are not established and they are subject of several investigations. Below, 4 possible mechanisms were considered.

Limonene and perillyl alcohol can exert chemopreventive actions such as cellular effects (induction of apoptosis), biochemical effects (such as the inhibition of isoprenylation of small G proteins), re-differentiation of tumor cells [overexpression of the transforming growth factor (TGF)-β], and inhibition of angiogenesis (52).

Monoterpenes can act during the all phases of carcinogenesis. During initiation phase, one effect is the prevention of interaction of carcinogens with DNA. In the promotion phase, the more accepted effect is the inhibition of cancer cell development. The more accepted roles of monoterpenes in later stages of carcinogenesis are induction of apoptosis, increase re-differentiation (47).

3.3.1. Brief description of some of the cellular effects of monoterpenes in cancer Some authors have already associated cancer prevention with increased rates of apoptosis. Induction of apoptosis is generally linked to other mechanisms involved in cancer prevention. Several effects caused by limonene and perillyl alcohol (like the inhibition of protein prenylation and induction of *c-Jun*, transforming growth factor-β (TGF-β), TGF-β receptor, and mannose-6-phosphate/insulin-like growth factor II receptor can influence apoptotic pathways (46). Monoterpenes can induct Phase 2 detoxification enzymes. Watson *et al.*

(46), in an interesting review, stated that limonene can promote these effects as consequences of the induction of apoptosis.

An investigation reported of the action of perillyl alcohol in liver cancer induced in male Fischer rats (53). The authors reported the monoterpene was able to increase apoptotic index. They also reported that perillyl alcohol treatment was able to reduce the tumors mass by a 10-fold factor, indicating that the monoterpene was really effective in inhibiting liver tumor growth. The apoptotic capacity of perillyl alcohol was accessed by using a TGF-β1-pre antibody. It was concluded that the exposure to the monoterpene during 19 days lead to a significant increase in apoptotic index: 5-fold for large tumors and 10-fold for small tumors compared to untreated animals.

More recently, Elegbede *et al*. (54) assessed the role of perillyl alcohol and perillyl aldehyde in proliferation of A549 (human lung adenocarcinoma) and BroTo (human squamous carcinoma of the tongue) carcinoma cell lines. The authors concluded that both monoterpenes were able to inhibit both cell lines proliferation according to a concentration-dependent relation. The IC_{50} values for BroTo cells were 1.0 and 3.2 mM to perillyl alcohol and perillyl aldehyde; and for A549 cells the values were 1.2 and 3.0 mM to perillyl alcohol and perillyl aldehyde (IC_{50} values were determined after 24 hr of cell exposure to the monoterpenes). Perillyl alcohol showed to be more effective than perillyl aldehyde according to the IC_{50} values.

Investigations related to the oncogene Bcr/Abl seems to be important because this gene can play a role in the pathogenesis of some leukemia as it enable factor-independent proliferation and make the leukemia cells more resistant to apoptosis (55).

In this way, a recent research reported the possible anti-leukemia role of perillyl alcohol in Bcr/Abl-transformed cells (56). The authors investigated the possible anti-leukemia effect of the monoterpene in interfere with Ras prelylation and in signaling Raf, Mek, and Erk kinases in Bcr/Abl-transformed cells. These kinases work in cascade and play an important role in human cancer and leukemia. The authors reported that the monoterpene induced growth arrest and apoptosis, did not inhibit protein prenylation, did not affect Bcr/Abl kinase and Raf activities.

Later, the same author reported the action of perillyl alcohol in the c-Myc-dependent apoptosis in Bcr/Abl-transformed leukemia cells (55). The author reported that the apoptosis promoted by the monoterpene is actually cause by a cell growth arrest. He also stated that perillyl alcohol showed 'specific cytotoxic activity against Bcr/Abl-transformed leukemia cells'; however, IC_{50} of the monoterpene showed to be about 300-500 mM, indicating that it is a weak agent, as commented by the author.

More recently, Wiseman *et al*. (57) evaluated cellular effects of perillyl alcohol in combination with 2 other terpenoids (*trans*-farnesol and geraniol) on human pancreatic adenocarcinoma cells. Some of the results obtained with the assays with perillyl alcohol are reported below. The authors concluded that perillyl alcohol can act through antiproliferative mechanism of action to cause G1 arrest in human pancreatic adenocarcinoma cells. They revealed also that the application perillyl alcohol resulted in chemopreventive and chemopreventive actions, causing arrest in G0/G1 phase of cell cycle by the induction of inhibitors from cyclin kinase, resulting in a decrease in expression of downstream cell cycle-related proteins.

3.3.2. Brief description of some of the biochemical effects of monoterpenes in cancer Paduch *et al*. (47) stated that the most important anticancer effect of monoterpenes is the role on the post-translational isoprenylation of proteins regulating the growth of cells (prenylated proteins are recognized to regulate cell growth and transformation).

Such isoprenylation inhibitions could alter signal transduction and result in altered gene expression. The results of a new gene expression screen-subtractive display-have identified or confirmed several up- or down-regulated genes in regressing mammary carcinomas. For example, these regressing tumors overexpress the mannose 6-phosphate/IGF II receptor. The product of this gene both degrades the mammary tumor mitogen IGF II and activates the cytostatic factor TGF-β. These and other alterations in the gene expression of mammary carcinomas lead to a G1 cell cycle block, followed by apoptosis, redifferentiation, and finally complete tumor regression in which tumor parenchyma is replaced by stromal elements (45).

Cox (58) stated that the antitumour effect of limonene and perillyl alcohol is probably due to the interaction with GGTase (geranylgeranyltransferase), which make these compounds able to block the prenylation of cell-growth regulating proteins, not only Ras, but also from Rho family. Paduch *et al.* (47) stated that the activity of limonene and its oxygenated derivatives can be related to the selective inhibition of the post-translational isoprenylation oncoprotein p21ras regulating signal transduction and cell growth. The authors stated also that this effect has also a role on gene expression, apoptosis, cellular re-differentiation, which can lead to tumor regression.

Pancreatic cancer is one of the most lethal cancers. Lebedeva *et al*. (59) investigated the chemopreventive effect of a combination of perillyl alcohol with adenovirus-mediated mda-7/IL-24 (Ad.mda-7) on pancreatic cancer induced in mice. The mice were treated with various aggressive pancreatic cell lines carrying both wild-type and mutant K-ras in order to evaluate the combinatorial treatment on the inhibition of cancer cells development. The authors tested the viability of normal cells in presence of perillyl alcohol and concluded that pancreatic carcinoma cells were significantly more sensitive to the monoterpene than normal cells (IC_{50} of perillyl alcohol was 300-500 and 600 mmol/L for pancreatic carcinoma cells and normal cells, respectively). The authors reinforce that the treatment with the combination of perillyl alcohol and Ad.mda-7 was more effective than the treatment with them alone. Similarly, the authors also found that perillyl alcohol overrides the 'translational block of mda-7/IL-24 mRNA' in pancreatic cells (the MDA-7/IL-24 protein expression was detected only in treatments with perillyl alcohol and Ad.mda-7). The tumors found in mice treated with perillyl alcohol were smaller in size compared with control animals. When the perillyl alcohol treatment was stopped, the tumors start proliferating; furthermore, the authors reported that the treatment with the monoterpene resulted in significant increase in the survival of the mice compared with the

control group. Apoptosis was also induced in the treatments with the combination of perillyl alcohol and Ad.mda-7.

3.3.3. Brief description of some of differential gene regulation effects of monoterpenes in cancer Mills *et al.* (53) evaluated the role of perillyl alcohol in the expression of mRNA levels for mannose 6-phosphate/ insulin-likegrowth factor II receptor and the TGF-β type I, II, and Ill receptors. They found that the treatment with monoterpene increased liver tumor mRNA expression for M6P/IGF-II receptors. They reported also that the rats treated with monoterpene did not show increased levels of mRNA for the TGF-β type II and III receptors in normal tissue compared to untreated rats. On another way, treated rats showed elevated levels of TGF-β type I (100% increase), II (38% increase), and III (49% increase) receptors in large tumors in comparison to untreated animals.

Later, the same research group from USA has reported the effect of limonene on gene expression in mammary carcinomas (60). The authors evaluated the effect of limonene on the treatment of the DMBA-induced carcinoma in virgin female Wistar rats at 50-55 day of age. The DNA of carcinomas cells was amplified. The RNA expression of lipocortin, transforming growth factor β type II receptor and neuroligin 1 were determined by competitive reverse transcriptase-polymerse chain reaction (RT-PCR). The authors found that the subtractive display screen methodology was able to identify 42 monoterpene-induced genes and 58 monoterpene-repressed genes. The authors concluded that some of the gene identified could play a role on the transforming growth factor β signal transduction pathway. They concluded this after the isolation of the mannose 6-phosphate/insulin-like growth factor II receptor (associated with the regression of carcinomas) and the transforming growth factor β type II receptor (a recognized tumors suppressor gene). Another gene induced by limonene was lipocortin 1, a marker for apoptosis during mammary gland involution. The results showed by this investigation reinforce that limonene can stimulate apoptosis and differentiation with clear effects on the carcinoma regression.

TGF-β activation can be mediated by perillyl alcohol, what is related to improved mRNA synthesis encoding TGF-β receptors. This activation is also associated with the induction of apoptosis. Perillyl alcohol can induce the production of cyclin and may inhibit the synthesis of coenzyme Q (CoQ is related to mitochondrial respiratory metabolism); both leading to the induction of apoptosis of tumor cells (61).

Another recent investigation reported the role of perillyl alcohol on the expression of Ras-related protein (62). The authors used K562 cells in presence and absence of mevalonate depletion by using Lov, an HMG-CoA reductase inhibitor. The results showed that both the perillyl alcohol enantiomers were able to decrease Ras and RhoA according to a concentration-dependent response. Decreases of 51-67 and 33-39% for Ras and RhoA, respectively, were obtained with the monoterpene concentrations of 0.5 mM. The same concentration of perillyl alcohol resulted in significant depletion of mevalonate, decreasing the up-regulation of Ras proteins. The authors examined the role of other monoterpenes on Ras and Ras-protein expression. No effects were found in the assays performed with limonene, limonene-oxide; perillic acid showed a small effect, whilst perillaldehyde, myrtenol, myrtanol, menthol carveol, and dihydrocarveol showed to be more effective on decreasing Ras-related proteins. In order to prove that the general effects of monoterpenes on diminished cell proliferation and general roles on protein synthesis and degradation did not play a role on the results, the authors performed 2 assays to corroborate that the effects of the monoterpenes evaluated were specific and were independent of the above-mentioned effects. In the first assay, they accessed the role of the monoterpenes on cell proliferation using the [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (MTT) assay and concluded that there was no correlation between expression of Ras-related proteins and MTT activity. In the second assay, the authors evaluated the role of the monoterpenes using [^{35}S] methionine pulse and pulse-chase experiments. They concluded that the monoterpenes "did not significantly alter the incorporation of [^{35}S] methionine into total protein pools" (62).

3.3.4. The potential of monoterpenes in the inhibition of angiogenesis An effective progress of angiogenesis is considered to be one of the conditions to the solid tumors growth. Thus, antiangiogenic therapies could be possible alternatives to cancer control (63). In this way, some researches are devoted in discovering new compounds that can prevent angiogenesis and act as antitumor agents. To the best of our known, few papers have related the effect of monoterpenes on angiogenesis. However, a recent investigation evaluated the potential of the monoterpene perillyl alcohol on the angiogenesis process (63). In order to access the angiogenic potential of perillyl alcohol, the authors analyzed the role of the terpene in the growth of new blood vessels in chicken embryo chorioallantoic membrane '*in vivo*', morphological differentiation of endothelial cells, endothelial cell proliferation and apoptosis and production of angiogenic growth factors. Perillyl alcohol was found to diminish blood vessels growth according to the chorioallantoic membrane assay, and showed also to inhibit differentiation of endothelial cells into capillary-like networks. Perillyl alcohol also induced apoptosis of endothelium cells, and inhibited the release of vascular endothelial growth factor (VEGF) and stimulated angiopoietin 2 (another protein growth factor that promotes angiogenesis); the authors commented that the inhibition of VEGF and production of angiopoietin 2 provides another perillyl alcohol regression tumor mechanism: the destruction of vascular networks that supports the tumor mass.

4. Conclusion

Here, the limonene and their derivatives are addressed in a particular way. They are considered as flavor compounds that can be obtained using biotransformation processes. This condition make these flavor compounds special as they are regarded as natural compounds, which make them interesting according to a marketing view, as the new trends point that the consumption of natural products tends to increase in detrimental to food ingredients obtained by chemical synthesis.

Furthermore, several researches indicate that these compounds

can be considered as more than flavor compounds. These monoterpenes can express some important biological features, such as bactericidal activity, induction of the immune response and action against some diseases.

Many research groups have reported the antitumor action of monoterpenes, especially perillyl alcohol. Several mechanisms are proposed, but there is not a consensus about which of them is the most important. Some authors consider that the monoterpenes can act according to several antitumor mechanisms at the same time, although it is not a consensus.

There are some research groups that are currently testing some biological actions of extracts of biotransformation of terpenes. Our Brazilian group is one of them, and some results are promising. Some '*in vitro*' assays have already showed good results. However, much work is needed to provide consistent data to prove the correct actions of these promising compounds in the human health, especially in terms of diseases prevention.

References

1. U.S. Ag, LLC-100% Nature Friendly Agriculture. Available from: http://www.unitedstatesag.org. Accessed on Sep. 9, 2008.
2. Duetz WA, Bouwmeester H, Beilen JB, Witholt B. Biotransformation of limonene by bacteria, fungi, yeasts, and plants. Appl. Microbiol. Biot. 61: 269-277 (2003)
3. Carvalho CCCR, Fonseca MMR. Biotransformation of terpenes. Biot. Adv. 24: 134-142 (2006)
4. Dhavalikar RS, Bhattacharyya PK. Microbiological transformations of terpenes. VIII. Fermentation of limonene in a soil pseudomonad. Ind. J. Biochem. 3: 144-157 (1966)
5. Dhavalikar RS, Rangachari PN, Bhattacharyya PK. Microbiological transformations of terpenes. IX. Pathways of degradation of limonene in a soil pseudomonad. Ind. J. Biochem. 3: 158-164 (1966)
6. Maróstica-Jr MR, Pastore GM. Production of R-(+)-α-terpineol by the biotransformation of limonene from orange essential oil, using cassava waste water as medium. Food Chem. 101: 345-350 (2007)
7. Braddock RJ, Cadwallader KR. Bioconversion of citrus *d*-limonene. pp. 142-148. In: Fruit Flavors: Biogenesis, Characterization, and Authentication. Rouseff RL, Leahy MM (eds). ACS Symp. Series No. 596. American Chemical Society, Washington, DC, USA (1995)
8. Maróstica-Jr MR, Pastore GM. Biotransformation of limonene: A review of the main metabolic pathways. Quim. Nova 30: 382-387 (2007)
9. Crowell PL. Prevention and therapy of cancer by dietary monoterpenes J. Nutr. 129: 775S-778S (1999)
10. Van der Werf MJ, Swarts HJ, De Bont JAM. *Rhodococcus erythropolis* DCL14 contains a novel degradation pathway for limonene. Appl. Environ. Microb. 65: 2092-2102 (1999)
11. Van der Werf, MJ, Keijzer PM, Van der Schaft PH. *Xanthobacter* sp C20 contains a novel bioconversion pathway for limonene. J. Biotechnol. 84: 133-143 (2000)
12. University of Minnesota. Biocatalysis, biodegradation Database. Available from: http://umbbd.msi.umn.edu/. Accessed on Sep. 9, 2008.
13. Speelmans G, Bijlsma A, Eggink G. Limonene bioconversion to high concentrations of a single and stable product, perillic acid, by a solvent-resistant *Pseudomonas putida* strain. Appl. Microbiol. Biot. 50: 538-544 (1998)
14. Chatterjee T, Bhattacharyya DK. Biotransformation of limonene by *Pseudomonas putida*. Appl. Microbiol. Biot. 55: 541-546 (2001)
15. Noma Y, Yamasaki S, Asakawa Y. Biotransformation of limonene and related compounds by *Aspergillus cellulosae*. Phytochemistry 31: 2725-2727 (1992)
16. Trytek M, Fiedurek J. A novel psychrotrophic fungus, *Mortierella minutissima*, for *d*-limonene biotransformation. Biotechnol. Lett. 27: 149-153 (2005)
17. Marostica-Jr MR, Mota NO, Baudet N, Pastore GM. Fungal biotransformation of monoterpenes found in agroiindustrial residues from orange and pulp industries into aroma compounds: Screening using solid phase microextraction. Food Sci. Biotechnol. 16: 37-42 (2007)
18. Abraham WR, Stumpf B, Kieslich K. Microbial transformations of terpenoids with 1-*p*-menthene skeleton. Appl. Microbiol. Biot. 24: 24-30 (1986)
19. Demyttenaere JCR, van Belleghem K, De Kimpe N. Biotransformation of (*R*)-(+)- and (*S*)-(-)-limonene by fungi and the use of solid phase microextraction for screening. Phytochemistry 57: 199-208 (2001)
20. Onken J, Berger RG. Biotransformation of citronellol by the basidiomycete Cystoderma carcharias in a aerated-membrane bioreactor. Appl. Microbiol. Biot. 51: 158-163 (1999)
21. Bowen ER. Potential by-products from microbial transformation of D-limonene. Florida State Horticult. Soc., USA 88: 304-308 (1975)
22. Lupien S, Karp F, Wildung M, Croteau R. Regiospecific cytochrome P450 limonene hydroxylases from mint (mentha) species: cDNA isolation, characterization, and functional expression of (-)-*4S*-limonene-3-hydroxylase and (-)-*4S*-limonene-6-hydroxylase. Arch. Biochem. Biophys. 368: 181-192 (1999)
23. Vank T, Valterová I, Vaková R, Vaisar T. Biotransformation of (-)-limonene using *Solanum aviculare* and *Dioscorea deltoidea* immobilized plant cells. Biotechnol. Lett. 21: 625-628 (1999)
24. Duetz WA, Fjällman AHM, Ren S, Jourdat C, Witholt B. Biotransformation of D-limonene to (+) *trans*-carveol by toluene-grown *Rhodococcus opacus* PWD4 cells. Appl. Environ. Microb. 67: 2829-2832 (2001)
25. Kraidman G, Mukherjee BB, Hill ID. Conversion of D-limonene into an optically active isomer of α-terpineol by a *Cladosporium* species. Bacteriol. Proc. 69: 63 (1969)
26. Tan Q, Day DF, Cadwallader KR. Bioconversion of (*R*)-(+)-limonene by *P. digitatum* (NRRL 1202). Process Biochem. 33: 29-37 (1998)
27. Tan Q, Day DF. Bioconversion of limonene to α-terpineol by immobilized *Penicillium digitatum*. Appl. Microbiol. Biot. 49: 96-101 (1998)
28. Tan Q, Day DF. Organic co-solvent effects on the bioconversion of (*R*)-(+)-limonene to (*R*)-(+)-alpha-terpineol. Process Biochem 33: 755-761 (1998)
29. Adams A, Demyttenaere JCR, De Kimpe N. Biotransformation of (*R*)-(+)- and (*S*)-(-)-limonene to α-terpineol by *Penicillium digitatum* - investigation of the culture conditions. Food Chem. 80: 525-534 (2003)
30. Maróstica-Jr MR, Pastore GM. Biotransformation of citronellol in rose-oxide using cassava wastewater as a medium. Ciênc. Tecnol. Aliment. 26: 609-696 (2006)
31. Bicas JL, Barros FFC, Wagner R, Godoy HT, Pastore GM. Optimization of *R*-(+)-α-terpineol production by the biotransformation of *R*-(+)-limonene. J. Ind. Microbiol. Biot. 35: 1061-1070 (2008)
32. Bakkali F, Averbeck S, Averbeck D, Idaomar M. Biological effects of essential oils - A review. Food Chem. Toxicol. 46: 446-475 (2008)
33. Turina AV, Nolan MV, Zygadlo JA, Perillo MA. Natural terpenes: Self-assembly and membrane partitioning. Biophys. Chem. 122: 101-113 (2006)
34. Soylu EM, Soylu S, Kurt S. Antimicrobial activity of the essential oils of various plants against tomato late blight disease agent *Phytophthora infestans*. Mycopathology 161: 119-128 (2006)
35. Högnadóttir A, Rouseff RL. Identification of aroma active compounds in orange essence oil using gas chromatography-olfactometry and gas chromatography-mass spectrometry. J. Chromatogr. A 998: 201-211 (2003)
36. Selli S, Cabaroglu T, Canbas A. Volatile flavour components of orange juice obtained from the cv. Kozan of Turkey. J. Food Compos. Anal. 17: 789-796 (2004)
37. Sonboli A, Eftekhar F, Yousefzadi M, Kanani MR. Antibacterial activity and chemical composition of the essential oil of *Grammosciadium platycarpum* Boiss. from Iran. Z. Naturforsch C 60: 30-34 (2005)
38. Mourey A, Canillac N. Anti-*Listeria monocytogenes* activity of

essential oils components of conifers. Food Control 13: 289-292 (2002)
39. Van Sonsbeck HM, Beeftink HH, Tramper J. Two-liquid phase bioreactors. Enzyme Microb. Tech. 15: 722-729 (1993)
40. López-Varela S, González-Gross M, Marcos A. Functional foods and the immune system: A review. Eur. J. Clin. Nutr. 56: S29-S33 (2002)
41. Raphael TJ, Kuttan G. Immunomodulatory activity of naturally occurring monoterpenes carvone, limonene, and perillic acid. Immunopharm. Immunot. 25: 285-294 (2003)
42. Evans DL, Miller DM, Jacobsen KL, Bush PB. Modulation of immune responses in mice by *d*-limonene. J. Toxicol. Env. Health 20: 51-66 (1987)
43. Hamada M, Uezu K, Matsushita J, Yamamoto S, Kishino Y. Distribution and immune responses resulting from oral administration of *d*-limonene in rats. J. Nutr. Sci. Vitaminol. 48: 155-160 (2002)
44. Del Toro-Arreola S, Flores-Torales E, Torres-Lozano C, Del Toro-Arreola A, Tostado-Pelayo K, Ramirez-Dueñas MG, Daneri-Navarro A. Effect of *d*-limonene on immune response in BALB/c mice with lymphoma. Int. Immunopharmacol. 5: 829-838 (2005)
45. Gould MN. Cancer chemoprevention and therapy by monoterpenes. Environ. Health Persp. 105: 977-979 (1997)
46. Watson WH, Cai J, Jones DP. Diet and apoptosis. Annu. Rev. Nutr. 20: 485-505 (2000)
47. Paduch R, KandeferSzerszeñ M, Trytek M, Fiedurek J. Terpenes: Substances useful in human healthcare. Arch. Immunol. Ther. Ex. 55: 315-327 (2007)
48. Samaila D, Toy BJ, Wang RC, Elegbede AJ. Monoterpenes enhanced the sensitivity of head and neck cancer cells to radiation treatment *in vitro*. Anticancer Res. 24: 3089-3095 (2004)
49. Da Fonseca CO, Masini M, Futuro D, Caetano R, Gattass CR, Quirico-Santos T. Anaplastic oligodendroglioma responding favorably to intranasal delivery of perillyl alcohol: A case report and literature review. Surg. Neurol. 66: 611-615 (2006)
50. Da Fonseca CO, Landeiro JA, Clark SS, Quirico-Santos T, Carvalho MGC, Gattass CR. Recent advances in the molecular genetics of malignant gliomas disclose targets for antitumor agent perillyl alcohol. Surg. Neurol. 65: 2-9 (2006)
51. Da Fonseca CO, Schwartsmann G, Fischer J, Nagel J, Futuro D, Quirico-Santos T, Gattass CR. Preliminary results from a phase I/II study of perillyl alcohol intranasal administration in adults with recurrent malignant gliomas. Surg. Neurol. 70: 259-267 (2008)
52. Bailey HH, Attia S, Love RR, Fass T, Tutsch, RCK, Harris L, Jumonville A, Hansen R, Shapiro GR, Stewart JA. Phase II trial of daily oral perillyl alcohol (NSC 641066) in treatment-refractory metastatic breast cancer. Cancer Chemoth. Pharm. 62: 149-157 (2008)
53. Mills JJ, Chari RS, Boyer IJ, Gould MN, Jirtle RL. Induction of apoptosis in liver tumors by the monoterpene perillyl alcohol. Cancer Res. 55: 979-983 (1995)
54. Elegbede JA, Floresa R, Wang RC. Perillyl alcohol and perillaldehyde induced cell cycle arrest and cell death in BroTo and A549 cells cultured *in vitro*. Life Sci. 73: 2831-2840 (2003)
55. Clark SS. Perillyl alcohol induces c-Myc-dependent apoptosis in Bcr/Abl-transformed leukemia cells. Oncology 70: 13-18 (2006)
56. Clark SS, Zhong L, Filiault D, Perman S, Ren Z, Gould M, Yang X. Anti-leukemia effect of perillyl alcohol in Bcr/Abl-transformed cells indirectly inhibits signaling through Mek in a Ras- and Raf-independent fashion. Clin. Cancer Res. 9: 4494-4504 (2003)
57. Wiseman DA, Werner SR, Crowell PL. Cell cycle arrest by the isoprenoids perillyl alcohol, geraniol, and farnesol is mediated by $p21^{Cip1}$ and $p27^{Kip1}$ in human pancreatic adenocarcinoma cells. J. Pharmacol. Exp. Ther. 320: 1163-1170 (2007)
58. Cox AD. Farnesyltransferase inhibitors: Potential role in the treatment of cancer. Drugs 61: 723-732 (2001)
59. Lebedeva IV, Su ZZ, Vozhilla N, Chatman L, Sarkar D, Dent P, Athar M, Fisher PB. Chemoprevention by perillyl alcohol coupled with viral gene therapy reduces pancreatic cancer pathogenesis. Mol. Cancer Ther. 7: 2042-2050 (2008)
60. Ariazi EA, Gould MN. Identifying differential gene expression in monoterpene-treated mammary carcinomas using subtractive display. J. Biol. Chem. 271: 29286-29294 (1996)
61. Ahn KJ, Lee CK, Choi EK, Griffin R, Song CW, Park HJ. Cytotoxicity of perillyl alcohol against cancer cells is potentiated by hyperthermia. Int. J. Radiat. Oncol. 57: 813-819 (2003)
62. Holstein SA, Hohl RJ. Monoterpene regulation of Ras and Ras-related protein expression. J. Lipid Res. 44: 1209-1215 (2003)
63. Loutrari H, Hatziapostolou M, Skouridou V, Papadimitriou E, Roussos C, Kolisis FN, Papapetropoulos A. Perillyl alcohol is an angiogenesis inhibitor. J. Pharmacol. Exp. Ther. 311: 568-575 (2004)