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Major bioactivities and mechanism of action of essential oils and their components

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ABSTRACT: Essential oils are gaining increasing interest for their antimicrobial and antiviral properties, as well as for their preventive and therapeutic actions against many human pathologies. Herein, we present an overview on new discoveries in essential oil research, discussing antimicrobial activity, as well as immunomodulatory, anti-apoptotic, anti-angiogenic and anti-tumoural properties. In addition, we emphasize recent advances in the identification of bioactive components and understanding of their mechanism of action. We discuss their molecular diversity and wide spectrum of activity as well as their structure–activity relationships and capability of targeting paradoxical responses triggered by different genes and pathways. Finally, we emphasize the effort required to isolate and identify the bioactive components of essential oils and to determine their cytotoxicity as their specificity. Thus, new approaches to specifically address bioactive components to selected targets could enhance the latter property in order to accommodate any cytotoxicity towards dysfunctioning loci.

Keywords: essential oils; antimicrobial activities; immuno-modulatory; anti-tumour; anti-apoptotic and anti-angiogenic properties

Introduction

Currently, several questions are raised concerning safety and the ratio of benefits to side effects of compounds used in medicine or in the food industry. Moreover, the extensive use of antibiotics in human medication as well as in animal farms, is leading to the emergence of resistant bacterial strains. Additionally, a growing number of allergic cases (allergies following absorption through the oral route, and others observed via dermal applications) in the modern world has become a real health problem.[1] Therefore, it is necessary to find alternative treatments that can fight bacteria resistant to antibiotics and heal or at least alleviate allergic reactions. Thus, two strategies can be undertaken to achieve these two objectives: (1) development of a new generation of antibiotics, and (2) search for natural products whose antibacterial, antifungal and antiviral properties have been known for centuries in the field of alternative and popular medicines. Scientific evidence for these and other properties, discussed below, are already available, which has encouraged the increasing integration of natural products into modern medicine.[2]

Among the natural products found in aromatic plants are known secondary metabolites and essential oils. Essential oils are volatile liquids, or semi-liquids, extracted from plants, usually by steam vaporization and cold-press techniques. However, contrary to what was mentioned in a 2000 review on the biological activity of essential oils and their constituents,[3] solvent extraction, simultaneous distillation–extraction (SDE) and supercritical fluid extraction (SFE) are not methods for producing essential oils, by definition. Indeed, using such methods would lead to the extraction and identification of many bioactive compounds that are not usual constituents of essential oils. Essential oils are complex mixtures of monoterpene and sesquiterpene hydrocarbons (10 and 15 carbon atoms, respectively) and their oxygenated derivatives (alcohols, aldehydes, ketones) as well as phenylpropanoids. In some cases, essential oils may encompass other chemical families like fatty acids, oxides and sulfur derivatives. Essential oils have gained interest as potential sources for bioactive natural molecules and are the subject of studies for their possible use as alternative medications for the treatment of infectious diseases. The long known antimicrobial actions of essential oils are now being extensively scientifically reviewed and applied in health and industry fields.[4] Various *in vitro* studies have confirmed the inhibitory action of essential oils against bacteria, fungi, yeasts, viruses and protozoa.[5–7] Many regions, such as the Mediterranean region, are rich in aromatic plants, and production of essential oils from these plants can be a profitable source for the economic and ecological development for these countries.

In this review, we describe methods of analysis of the antibacterial activity of essential oils. Following, we will review the published data on antimicrobial activity of essential oils, and discuss the structure–activity relationships and mechanisms of action of their active components. Finally, an overview about other preventive and therapeutic properties of essential oils, such as anti-inflammatory, immunomodulatory, anti-angiogenic, anti-tumour and pro-apoptotic properties will be given and the mechanisms of action and putative target pathways discussed. Other biological activities of essential oils and their components, including anti-allergic, enzyme inhibitory, psychological, antimutagenic, antiviral, insect repellent and molluscicidal, as well as various uses and applications of essential oils are already argued[3] and are not discussed in this review.

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Methods of Analysis of the Antimicrobial Activity of Essential Oils

The methods for evaluating the antimicrobial activity of antibiotics and disinfectants are well established and in many cases, standardized. These methods were developed for hydrophilic antimicrobials such as antibiotics and require several modifications in order to test the hydrophobicity of products such as essential oils.[6] Classical techniques for the analysis of the essential oil antimicrobial activity included agar diffusion and broth or agar dilution methods. The advances in 'omics' analysis opened the way for the use of novel techniques to assess the antimicrobial activity of essential oils and their components.

The Agar Diffusion Method[6]

Diagnostic laboratories commonly use dissemination methods or standard antibiograms. Blotting-paper discs impregnated with antibiotics are deposited on the surface of an agar medium previously inoculated with a pure culture of the studied strain. Upon application, antibiotics spread uniformly and their concentrations are inversely proportional to the distance from the disk (Figure 1A). After incubation, discs with active molecules will be surrounded by circular inhibition zones corresponding to the absence of growth. In standard conditions, the diameter of the inhibition zone would depend only on the sensitivity of the microorganism. On the edge of the inhibition zone, the antibiotic concentration in the agar gel would be equal to the minimum inhibitory concentration (MIC). Dissemination methods do not directly quantify this value. However, there is a logarithmic (log 2 base) relationship between the diameter of the inhibition zone and the MIC measured by diffusion. This relationship, called line of regression,[5] was established by specialized laboratories working under standardized conditions. Changes in the diffusion method include forming wells in the agar or the use of 96-well plates.[8] These are the methods of choice for many researchers to evaluate antimicrobial activity of essential oils. The size of the inhibition zone formed around the well indicates the relative activity of the sample. However, addition factors, including the volume and the medium used, the concentration and age of inoculum, incubation conditions and the size, polarity and the conformation of the active ingredient, may affect final results. The agar diffusion method has been standardized and used to test antibiotics. Nevertheless, some disadvantages were reported when using this method to test essential oils. This is mainly due to the hydrophobic nature of essential oils. An example of these disadvantages is the possibility of obtaining inconsistent results and a low correlation with MIC values.[9] The hydrophobic nature of the tea tree oil components, for example, limits their diffusion in agar. Only terpinen-4-ol, 1,8-cineole and α-terpineol, which have low solubility in water, can diffuse into the agar starting from the disk and the hydrocarbon components either remain on the disc or evaporate. Consequently, the contribution of these components to the activity of the essential oil cannot be evaluated, and there is possibility of under-estimation. Therefore, the usefulness of the agar diffusion method is limited only to the generation of qualitative and preliminary results since the hydrophobic nature of some essential oils prevents the uniform distribution of the active substances in the agar.[9]

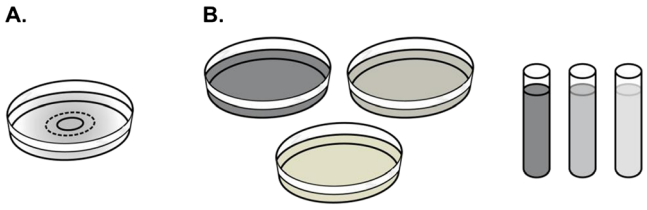


Figure 1. Common techniques for the analysis of the essential oil antimicrobial activity. (A) Agar diffusion method. The middle circle corresponds to the disc on which the antimicrobial agent is added. The surrounding dashed circle corresponds to the inhibition zone. The diffusion of the antimicrobial agent is represented by a radial gradient. On the edge of this zone, the antibiotic concentration in the agar gel would be equal to the minimum inhibitory concentration (MIC). (B) Broth (right) or agar (left) dilution method. The shadings on the plates and tubes represent the concentration of the antimicrobial agent

Dilution Method (Broth and Agar)

Dilution methods are performed in liquid or solid medium (Figure 1B). Media containing decreasing concentrations of antibiotic are inoculated with a standardized bacterial suspension, and the presence or absence of growth after incubation could be observed. The measure of MICs has been extensively used to quantify antimicrobial activity of essential oils and several variations of the method have been proposed.[6] The tested microorganism is added to serial dilutions of the agent (i.e. essential oil) prepared in a solid or liquid culture medium. The presence or absence of growth is then determined after incubation. The MIC would be the lowest concentration value of the agent for which there is no bacterial growth visible to the naked eye. The value of the MIC depends on the starting concentration chosen. An initial concentration is taken that is of two orders of magnitude, which allows obtaining a series of 1:2 dilutions, simple to express. The theoretical MIC would correspond to the median of the concentration range. The larger the concentration range is, the lower the precision of the MIC estimation.

Methods using the broth culture, particularly those carried out in microtitre wells, have the advantages of using low concentrations for a wide number of replicates and of using small volumes of the test agent and the culture medium. In these methods, turbidity[6] of the oil–water emulsion can interfere with the reading of the end point, particularly in the microtitre tests. For this reason, indicators such as fluorescein diacetate, *p*-iodonitrotetrazolium purple, triphenyl tetrazolium chloride[6] or resazurin[10] are used. Even so, problems arise with some indicators. For example, Carson *et al.*[11] report that the colour change of triphenyl tetrazolium chloride is not exactly correlated with the MIC. Dilution methods in agar exceed the turbidity and the indicator problems associated with the dilution methods in broth, and can be modified to be adapted to any microorganism by changing the culture medium and incubation conditions. Whatever the dilution method used, in broth or agar it is important to obtain a uniform dispersion of the essential oil to ensure a good contact with the microorganism, and therefore the reproducibility of the assay. In the absence of a solubilizing agent or emulsifier, some essential oils would separate from the broth or agar and would be visible as a layer on the surface of the medium. An ideal emulsifier must not affect, in any way, the tested microorganism and would not chemically interact with the components of the essential oil. In addition, it must have neither a synergistic[7] nor an antagonist effect on the essential oil activity.[5] Non-ionic emulsifiers such as Tween 20

and Tween 80 are relatively inactive when tested alone and, so far, widely reported,[6] with a recommended final concentration of 0.5% (v/v). However, at high concentration of essential oil, there is a risk of poor dispersion and therefore it remains essential to increase the concentration of Tween to gain better homogenization. Nevertheless, at high concentrations, the emulsifier can affect the MIC as for Tween, which can interfere by solubilizing the microorganism membrane.[10] For these reasons of standardization and interference, the results of studies using different concentrations of Tween 20 are not directly comparable.

'Omic' Techniques

Three main 'omic' techniques have been developed and used to analyse the genetic sequence profile (genomics),[12] the genetic expression profile (transcriptomics)[13,14] and the protein functional content (proteomics) of single species. However, metagenomics (microbial diversity), meta-transcriptomics and meta-proteomics would deal with entire microbial communities.[15] These techniques help to understand how communities respond to changes in their environment. While metagenomic studies are capable of providing a snapshot of the genetic composition of the community at any given time,[16] meta-transcriptomics and meta-proteomics studies are required to investigate the effect of rapid environmental changes on the abundance and composition of the active fraction of the community.[15] Similarly, transcriptomics and proteomics have emerged as the most adequate techniques to follow the antimicrobial activities of natural products, in general and of essential oils and their components, in particular on single pathogens.[17] These novel findings would become a prerequisite to understanding the mechanism of action of essential oils by identifying their targets and by identifying the disrupted molecular functions and pathways.[13,14] Moreover, the development of these techniques accelerates the search for active compounds since high throughput analyses and screens of a wide range of natural compounds against a wide number of pathogens (bacteria, fungi and viruses) would be possible. As a consequence, the use of the new 'omic' techniques will facilitate the selection of specific ligands that act on single cellular and molecular processes and so to obtain highly effective and safer drugs having fewer side effects.

Mass Spectrometry-based Techniques

The most applied analytical techniques are gas chromatography with flame ionization detection (GC-FID)[18] and gas chromatography–mass spectrometry (GC-MS).[19] They are used in essential oil therapy research to identify, compare and quantify their components. First, these techniques will allow an analysis of the effect of environmental conditions on essential oils composition. Consequently, they will open the way for the adjustment of essential oils compositions by varying one or several environmental parameters. In addition, the comparative analyses of composition and antimicrobial activity of several essential oils from various origins would be more significant since delicate changes in the concentration of the bioactive components can now be quantified. The MS-based techniques will allow a classification of essential oils based on their origin, on their concentration of bioactive components, and hence, on their antimicrobial activity potentials. The FID- and MS-based techniques will also be more useful once coupled to other techniques, such as the purification of the most concentrated components of essential oils and the analyses of their subsequent antimicrobial activity.[20] They can be also coupled to the 'omic' techniques, such as metabolomics, in order to perform quality control analyses on the composition of one essential oil, which has been exposed to changes in environment parameters. The coupling with metabolomic analysis, which is an important molecular phenotyping method for characterizing plant ecotypic variation,[21] will allow identification of chemical markers for discrimination and quality control among different groups of samples. Finally, coupling of FID- and MS-based techniques to transcriptomics and proteomics will facilitate the comparative analyses of the bioactive properties of essential oils and their components, giving the possibility to deduce hypotheses regarding the effect of changing the composition of essential oils on the transcriptome and/or the proteome of the cell. In addition, it will render the comparative analyses more significant, especially between essential oils or bioactive components with identical phenotypic impact since the omics techniques allows the identification of the targeted cellular particles and pathways. Accordingly, essential oils and their bioactive components can now be classified according to their genotypic rather to their phenotypic effects.

Antimicrobial Activity of Essential Oils and Mechanism of Action of their Bioactive Components

Antimicrobial Activity

Essential oils have many biological activities. In herbal medicine, they are used for their antiseptic properties against infectious diseases of bacterial and fungal origins. However, they also possess cytotoxic properties close to those of antiseptics and disinfectants that are used as antimicrobial agents with a broad spectrum of activity. In the two sectors of health and food safety, essential oils or their bioactive components could also be used as protective agents against phytopathogenic fungi and pathogenic microorganisms in food. Actually, there is much data reporting antimicrobial properties of essential oils of several kinds and from different origins. However, numerous publications give general results by simply indicating if the tested agent (an essential oil or one of its purified components) is bioactive or not against Gram-positive or Gram-negative bacteria or fungi. Most of the time, the authors give no details on the degree and spectrum of activity. Similarly, some publications only determine the relative activity of an essential oil by comparing the activity of different oils tested against the same microorganisms.[5,7] It has to be noted that the comparison of the bioactivity of essential oils with the same common name and the same major bioactive component may show significant divergence in the results. This is due to the difference in their general composition that can greatly influence the bioactivity of the essential oil. The main factors responsible for such divergence in the composition of essential oils are climate and environmental conditions. Furthermore, the method used to evaluate the antimicrobial activity and the choice of tested microorganisms varies from one laboratory to another.

Finally, results are often variable since several factors may vary between studies.[6] These include differences in microbial growth, concentration of the inoculum, solubility of the oil or its components or the use of an emulsifier and its concentration. As a consequence, there is a great need to develop a reliable standardized procedure to test antimicrobial effects of essential oils. Similarly, *in vivo* studies are needed to confirm *in vitro* results.

Antibacterial Activity

The antibacterial activity of essential oils has been widely studied. In two separate reports,[5,7] the results on the antimicrobial effects of essential oils obtained by two methods, diffusion and agar dilution, were compared. In each study, a large number of essential oils and/or their components were tested under the same conditions, making it easier to compare and interpret the results. The correlation between the antibacterial activity of tested components and their relative rates in the composition of essential oils, in addition to their chemical structures initiated several observations. Phenolic compounds such as carvacrol, eugenol and thymol from various plant origins[7] (Table 1 and Figure 2) are highly active against many microorganisms. The importance of the hydroxyl group in the phenolic structure was confirmed by comparing the activity of carvacrol with its methyl ether form. In addition, the relative position of the hydroxyl group influences the effectiveness of the terpenes as the two isomers, thymol and carvacrol (see Dorman and Deans for plant origin[7]), showed different activities against Gram-positive and Gram-negative bacteria. Furthermore, the importance of the phenolic ring was demonstrated by comparing the activity of thymol to *p*-cymene (Figure 2), a cyclic monoterpene hydrocarbon. The activity of *p*-cymene was found to be very weak.

The presence of an ester group in the structure of geranyl acetate (Figure 2) and bornyl acetate increases their activity against most tested microorganisms,[7] in comparison to the activity of their parent compounds (respectively, geraniol and borneol). However, many publications in the past showed an important activity of geraniol, compared to geranyl acetate, against many strains. It is their hydrophobic difference that may explain the above-mentioned results. It is known that alcohols have a bactericidal rather than a bacteriostatic activity against vegetative cells.[22] Terpene alcohols proved to be active against several microorganisms, and appeared to act by denaturing proteins. Other components, like ketones, have been tested. The presence of a carbonyl function in their chemical structure appeared to increase the antibacterial properties of terpenoids.[7] Thus it can be concluded that an increase in activity depends on the type of alkyl substituent incorporated in a non-phenolic cycle. An alkenyl substituent (1-methylethenyl) leads to a higher antibacterial activity as observed in the case of limonene [1-methyl-4-(1-methylethenyl) cyclohexene] (Table 1) with respect to an alkyl substituent (1-methylethyl) found in the structure of *p*-cymene [1-methyl-4-(1-methylethyl) benzene]. The introduction of a double bond[7] increases the activity of limonene compared to *p*-cymene mainly against Gram-negative bacteria, the most susceptible microorganisms. However, alkylation has been shown to increase the antibacterial activity of phenols. Based on these data, an allyl side chain seems to enhance the inhibitory effects of a component, especially against Gram-negative bacteria. Another element that has been shown to influence the bioactivity of essential oil components is their stereochemistry. It has been observed that α-isomers are relatively inactive compared to β-isomers (e.g. α-pinene) (Figure 2), and *cis*-isomers are inactive when compared to their trans-isomers counterparts (e.g. geraniol and nerol, Table 1). Finally, components with methyl isopropyl cyclohexane cycles are the most active, and the unsaturation of the cyclohexane ring increases more the antibacterial activity (e.g. terpinolene and terpineol, Figure 2).

Antifungal Activity

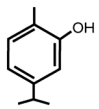
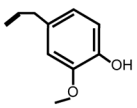
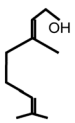
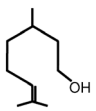
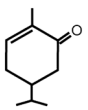
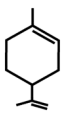
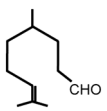
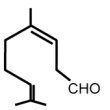
Given the complex chemotype composition of essential oils, there is the possibility to benefit from the synergy between components. However, some researchers favour studying an isolated component in order to later compare to the overall activity of the oil. When testing the fungi-static activity of essential oils in comparison to isolated components, it appears that this activity is directly related to the chemical composition of the essential oil. Moreover, as a result of the comparison of the activity of isolated aromatic components, the fungi-static activity appears to also depend on the presence of certain chemical functions within the tested components[23] (Table 1). As a conclusion, phenols (eugenol, chavicol and 4-allyl-2-6-dimethoxyphenol, Figure 2) are specifically more antifungal, even though acids (cinnamic and hydrocinnamic acids, Figure 2) also exhibit remarkable fungi-static properties. The methoxy groups, conversely, do not seem to significantly reinforce the fungi-static activity of the above-mentioned components (eugenol and 4-allyl-2-6-dimethoxyphenol). The isolated components can be classified according to their antifungal activity towards some fungi. This activity is estimated by duration of growth inhibition determined by a simple macroscopic observation. The antifungal activity decreases depending on the type of chemical functions: phenols $>$ cinnamic aldehydes alcohols $>$ aldehydes $\geq$ ketones $>$ ethers $>$ hydrocarbons.[24] Concerning phenolic components, the antifungal activity increases with the steric hindrance of the molecule (*p*-*n*-propylphenol thymol $<$ isoeugenol $<$ eugenol)[23] (Figure 2). The addition of alkyl groups to the benzene ring of phenol increases the antifungal property.[25] Therefore, a certain degree of hydrophobicity of phenolic components or aromatic aldehydes seems necessary to show an optimal antifungal characteristic.

Finally, it emerges that essential oils are effective against a broad spectrum of pathogenic and non-pathogenic microorganisms. Administered orally, these components can control a wide range of microbes although there is a risk of causing an imbalance in the intestinal microflora. The volatility and low solubility of essential oils and their components make them less interesting as disinfectants. However, they can be used for disinfecting rooms. In fact, the volatility property of essential oils could have the benefit of lowering microbial contamination in air and in inaccessible locations. Indeed, essential oils are also used in food as preservatives[26] and fragrance products as well as in perfumes and pharmaceuticals.

Mechanism of Action of the Bioactive Components of Essential Oils

Essential oils and their components have been studied for their antimicrobial activities (antibacterial, antifungal and antiviral); nevertheless, their mechanism of action remains poorly studied.

Table 1. Classification of aromatic molecules according to their chemical function

Aromatic component	Molecular structure	Physico-chemical characteristics	Content in some plants' essential oils (%)
Phenols	Examples  Carvacrol (CAS No. 499-75-2)	Density: 1.07 g/ml MW: 164.2 g/mol	Carvacrol in thyme [*Thymus vulgaris* (Lamiaceae)] 33% and in origan [*Origanum vulgare* (Lamiaceae)] 76%
	 Eugenol (CAS No. 97-53-0)	Density: 0.98 g/ml MW: 150.2 g/mol	Eugenol in clove [*Syzygium aromaticum* (Myrtaceae)] 82%, in bay rum tree [*Pimenta racemosa* (Myrtaceae)] 60% and in pepper [*Pimenta dioica* (Pimenta dioica)] 54%
Terpenic alcohols	Examples  Geraniol (CAS No. 106-24-1)	Density: 0.88 g/ml MW: 154.3 g/mol	Geraniol in palmarosa [*Cymbopogon martinii* (Poaceae)] 75–95%, in *Helichrysum* spp. (Asteraceae) 80–90%, in citronella java [*Cymbopogon winterianus* (Poaceae)] 12–18% and in *Cymbopogon nardus* (Poaceae) 20–40%
	 Citronellol (CAS No. 106-22-9)	Density: 0.86 g/ml MW: 156.3 g/mol	Citronellol in citronella java [*Cymbopogon winterianus* (Poaceae)] 11–15% and in *Cymbopogon nardus* (Poaceae) 10–20%
Cetones	Examples  Carvone (CAS No. 99-49-0)	Density: 0.96 g/ml MW: 150.2 g/mol	Carvone in caraway [*Carum carvi* (Apiaceae)] 50%
Aliphatic hydrocarbons, sesquiterpenes	Examples  Limonene (CAS No. 5989-54-8)	Density: 0.96 g/ml MW: 150.2 g/mol	Limonene in caraway [*Carum carvi* (Apiaceae)] 45%
Terpenic aldehydes	Examples  Citronellal (CAS No. 106-23-0)	Density: 0.89 g/ml MW: 154.3 g/mol	Citronellal in citronella java [*Cymbopogon winterianus* (Poaceae)] 35–45% and in lemon eucalyptus [*Eucalyptus citriodora* (Myrtaceae)] 90%
	 Neral and geranial (CAS No. 5392-40-5)	Density: 0.89 g/ml MW: 154.3 g/mol	Citral in lemongrass [*Cymbopogon citratus* (Poaceae)] 70–80% and in lemon balm [*Melissa officinalis* (Lamiaceae)] 50%

(Continues)

Table 1. *(Continued)*

Aromatic component	Molecular structure	Physico-chemical characteristics	Content in some plants' essential oils (%)
Ether oxides, peroxides	Examples  Cineole (CAS No. 470-82-6)	Density: 0.92 g/ml MW: 154.2 g/mol	Cineole in eucalyptus [*Eucalyptus globulus* (Myrtaceae)] 56%
	 Ascaridole (CAS No. 512-85-6)	Density: 1.01 g/ml MW: 168.2 g/mol	Ascaridole in epazote [*Chenopodium ambrosioides* (Amaranthaceae)] 61%

The table is adapted from Ochoa,[23] with modifications

The identification of active components of essential oils and understanding their mechanism of action, in addition to that of the essential oil mixture is essential. It will allow selecting the best and proper growing conditions, harvesting and extraction to obtain the ideal and most active composition of an essential oil. In addition, once the active component is known, it is then possible to develop and prepare synthetic analogs. These synthetic components would be better controlled in terms of preparation reproducibility and a higher economical viability.

It was suggested that essential oils, either inhaled or applied on the skin, act through their lipophilic fractions on the lipid moieties of cell membranes and, therefore, modify the activity of calcium and potassium ion channels.[27–30] Applied to a certain dose, essential oils saturate the membranes. They seem to interact with cell membranes depending on the structure and physicochemical properties of the components that can then affect the function of various membrane molecular structures[31]: transport systems, enzymes, ion channels[32,33] or receptors.

Several studies have been devoted to the different physiological effects of essential oils on the human body, e.g. stimulating, sedative and antidepressant effects. Similarly, some studies have focused on the effects of fragrance components on cognition, memory and mood. In these cases, the fragrance components are absorbed by inhalation and are able to cross the blood–brain barrier by interacting with receptors of the central nervous system.[34] Bioassays that attempted to describe and explain the action of essential oils are generally performed on mice, rats and frogs: e.g. the effect of peppermint oil on intestinal transport,[35] the study of the absorption of essential oils through the skin, the effect of some essential oils on skeletal muscle fibres[36] and the investigation of their analgesic properties.[37] Increasing numbers of physiotherapists and aromatherapists are currently using essential oils in private practice or hospitals and are publishing numerous positive medical effects in major aromatherapy journals.

In this section we will present the results of some studies that investigated the mechanism of action of essential oils and/or

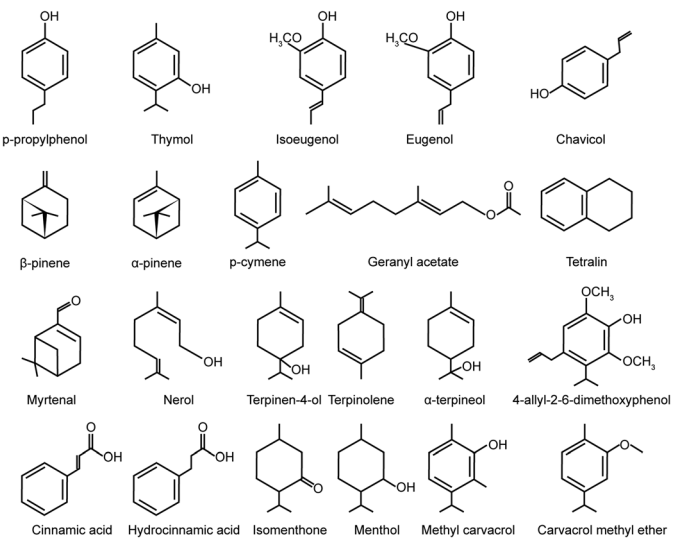


Figure 2. Selected terpene structures found in essential oils

their components supposed to be the active principles against different microorganisms. These studies focused on the mechanism of action of carvacrol on the food-borne bacterial pathogen *Bacillus cereus*, where the main role of the phenol group was revealed. We will then explain the mechanism of action of the essential oil of tea tree on *Staphylococcus aureus* bacterial cells.

Mechanism of Action of Carvacrol on the Food-borne Bacterial Pathogen *Bacillus cereus*: Role of the Phenol Group

Moderate food preservation technologies are becoming increasingly important in modern food industry. However, under these conditions, there is a serious risk on food security due to proliferation of spore-forming microorganisms. Among these microorganisms is the pathogenic bacterium *Bacillus cereus*, often associated with meat, fruits, vegetables, rice, milk and other dairy products. Levels of 1% to 20% of food-borne infections worldwide are caused by *Bacillus cereus*. A new method to reduce the proliferation of such microorganisms may be the use of essential oils. Antifungal and antibacterial effects of these components on different microorganisms have been described in several studies.[11,38–44] In the diverse group of essential oils components, there is a special consideration for carvacrol, which has strong antimicrobial properties. It is therefore used to preserve various foods, such as baked food products (up to 15.74 ppm), non-alcoholic beverages (up to 28.54 ppm/0.18 mM), and chewing gum (up to 8.42 ppm). Yet, the mechanism of action of this component remains unexplained. Hydrophobic molecules such as carvacrol are likely to have an influence on biological membranes. The cytoplasmic membrane of bacteria has two main functions: (1) a barrier and energy transduction function, which allows the membrane to form ionic gradients that can be used to carry out various processes; and (2) a matrix for the transmembrane proteins such as the F_0 complex of the ATP synthase.

Carvacrol found in the essential oil fractions of oregano [*Origanum vulgare* ssp. *hirtum* (Link) Letsw. (Lamiaceae)], and thyme [*Thymus vulgaris* L. (Lamiaceae)] was studied for its effects on the bioenergetic parameters of *Bacillus cereus* vegetative cells.[45] Incubation for 30 min in the presence of 1 to 3 mM of carvacrol exponentially reduced the number of viable bacterial cells. The use of 2 mM carvacrol significantly reduced the intracellular pool of ATP to a value close to zero in less than 7 min. Moreover, no proportional increase of extracellular ATP pool was observed. Depletion of internal ATP pool was associated with a change in membrane potential ($\Delta\Psi$). Thus, one can conclude that carvacrol does not enhance the membrane permeability for ATP, but the depletion of the internal ATP pool results only from reduction of ATP synthesis and/or increase ATP hydrolysis. This depletion of the ATP pool following the addition of lipophilic components was also observed in other studies.[44,45] Nevertheless, Helander *et al.*[46] observed a loss or leakage of ATP from cells exposed to carvacrol (2 mM). It is important to note that this study was performed on a Gram-negative bacterium, which has a different cell envelope.

Ultee *et al.*[45] showed that carvacrol concentrations greater than or equal to 0.01 mM, significantly reduces the $\Delta\Psi$, which was completely dissipated at concentrations equal or higher than 0.15 mM. Finally, an increase in the permeability of the cytoplasmic membrane for protons and potassium ions was observed at 0.25 and 1 mM of carvacrol, respectively. Gradients of soluble molecules along the cytoplasmic membrane using H^+ as coupling ion, can also be affected by the dissipation of the proton motive force. Consistent with these results, Sikkema and colleagues[47,48] showed an increase in the permeability of liposomal membranes to protons during exposure to tetralin, a synthetic molecule with one aromatic ring (Figure 2). Similarly, it has been previously noted an increase of protons influx, causing a dissipation of the ΔpH in the presence of ethanol. Furthermore, various studies have shown that the efflux of K^+ ions was a first indication of alteration of the bacterial membrane.[44,45] Heipieper *et al.*[49] observed a significant K^+ excretion to the outside environment during the exposure of *Pseudomonas putida* to phenol. Potassium is the major cytoplasmic cation for growing bacterial cells. It is also involved in various key functions in bacterial cells. K^+ plays a role in the activation of cytoplasmic enzymes, the maintenance of osmotic pressure and possibly, the regulation of cytoplasmic pH. The membrane potential, $\Delta\Psi$, depends primarily on cellular K^+ concentration. We can conclude from the study of the antibacterial activity of carvacrol that its activity depends on concentration and exposure time. Despite the absence of the immediate effect of carvacrol on the viability for concentrations up to 1 mM, clear effects on different bioenergetic parameters are observed. In particular, carvacrol interacts with the membranes of *Bacillus cereus* by changing their permeability to cations such as H^+ and K^+. The dissipation of ion gradients leads to an alteration or imbalance in the essential processes of the cell and ultimately to cell death.

It should be noted that carvacrol has biological effects at concentrations optimal to give flavour to food [e.g. non-alcoholic beverages (0.18 mM/28.54 ppm) and cooked foods (15.75 ppm)]. For products that can be infected with *Bacillus cereus*, carvacrol could be applied as an antimicrobial and as a flavour component. Ultee *et al.* subsequently demonstrated the role of the phenol group of carvacrol on *Bacillus cereus* viability.[44] The addition of carvacrol to a liposome suspension leads to an expansion of the liposome membrane. The investigation of the role of cymene, the biological precursor of carvacrol with no hydroxyl group, showed a higher affinity for liposomal membranes causing greater expansion. However, the effect of cymene on the membrane potential was less pronounced than the effect of carvacrol. In addition, cymene does not affect the pH gradient or the ATP pools.

A characteristic property of the hydroxyl group found on a phenol structure is its acidity, significantly higher than for a hydroxyl group found on an aliphatic structure. Carvacrol appears to act as a membrane transporter of monovalent cations by exchanging its hydroxyl proton to another ion such as K^+. Undissociated (protonated) carvacrol diffuses through the cytoplasmic membrane to the cytoplasm where it dissociates by releasing its proton. Then, it can bind and transport a K^+ ion (or another ion) from the cytoplasm, across the cytoplasmic membrane, to the outside environment. A proton is again fixed and the protonated carvacrol diffuses in a second time across the cytoplasmic membrane and releases again a proton into the cytoplasm. This assumption is based on the efflux of K^+ and the influx of H^+ observed in *Bacillus cereus* when exposed to carvacrol[44,45] (Figure 3). As the pK_a of the phenolic compounds is 10, 0.1% of carvacrol will be dissociated at the hydroxyl group in the experimental conditions at pH 7. Cymene does not have these properties as it is lacking the phenolic group. A large accumulation of cymene in the membrane probably causes an expansion of the membrane leading to a

passive diffusion of ions between the expanded phospholipids. However, the presence of the phenolic group appears to be more important for the antimicrobial activity than the only expansion of the membrane and therefore its destabilization. The hypothesis described above is confirmed by the results obtained with menthol, methyl carvacrol and thymol (Table 1 and Figure 2).[44] Although menthol has a hydroxyl group, it did not show a high antimicrobial activity. The reduced activity of menthol is not caused by the low partition of menthol in the membrane compared to that of carvacrol, but the lack of a system of delocalized electrons (double bonds or phenyl group) and therefore, the inability of the hydroxyl group to release its proton. Similarly, carvacrol methyl ether has no ability to release a proton and is therefore not an antimicrobial. Thymol, as carvacrol, contains a phenolic group and a system of delocalized electrons; therefore it has a strong antimicrobial activity. As a consequence, measurement of antibacterial activities of carvacrol, thymol, cymene, menthol and methyl carvacrol demonstrated that the hydroxyl group and the presence of a system of delocalized electrons are important elements for antibacterial activity.[44,45] In addition, several previous studies demonstrated the presence of a synergistic activity between carvacrol and cymene.[44,46,48] Presumably cymene acts synergistically with carvacrol by causing the expansion of the membrane resulting in its destabilization.

Mechanism of Action of the Essential Oil of *Melaleuca alternifolia* in *Staphylococcus aureus*

The essential oil of tea tree *Melaleuca alternifolia* Cheel (Myrtaceae Juss.) (tea tree oil, TTO) has a broad spectrum of antimicrobial activity. The mechanism of action of the TTO and its three main components, 1,8-cineole, terpinen-4-ol and α-terpineol (Figure 2), against *Staphylococcus aureus* has been studied previously.[11] Treatment with these components reduced cell viability of *S. aureus* in the stationary growth phase. From the analyses of TTO effect on *S. aureus* it has been concluded that the main target of this essential oil is not a macromolecular synthetic process. Moreover, none of the tested components caused any lysis (as followed by the absorbance at 620 nm) although the cells became disproportionately sensitive to subsequent autolysis. Unlike some antimicrobial agents (e.g. essential oils from oregano [*Origanum vulgare* ssp. *hirtum* (Link) Letsw. (Lamiaceae)], rosewood [*Dalbergia latifolia* (Fabaceae)] and thyme [*Thymus vulgaris* L. (Lamiaceae)]) significantly damaging the cytoplasmic membrane of a cell,[50] TTO is incapable of a sudden alteration of the cell wall. However, it induces the release of autolytic enzymes associated with the cell membrane, which may induce lysis.[11,51] Another hypothesis has been suggested for cell lysis that can be due to the osmotic pressure change.[11] The latter can indirectly disrupt the cytoplasmic membrane.

It has been observed that TTO, in addition to some of its components induced the loss of 260 nm absorbing material, suggesting a leakage of nucleic acids across the cytoplasmic membrane. Similar cytoplasmic losses are observed in the case of *E. coli* treated with lemongrass oil. TTO treatment triggered a loss of tolerance to NaCl, which correlated with the loss of absorbent materials at 260 nm. The former effect as well as the loss of tolerance to toxic components can be used as an index of membrane damage.

Other Bioactive Properties of Essential Oils

In addition to their antimicrobial and antiviral properties, essential oils and their active components have proven their efficiency on modulating the activity of some essential molecular mechanisms and signalling pathways in eukaryotic cells. For example, α-pinene inhibits respiratory activity in yeast

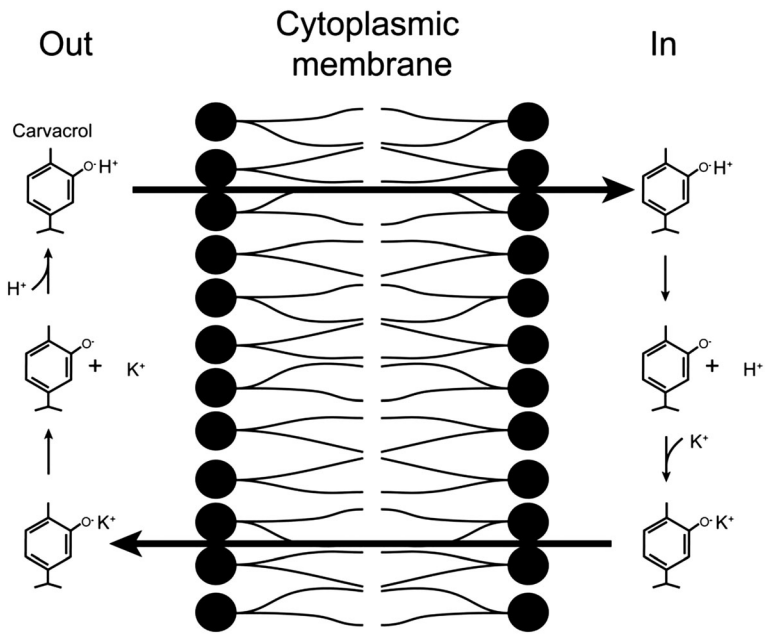


Figure 3. Schematic representation explaining the hypothesis of carvacrol mechanism of action. Undissociated carvacrol diffuses through the cytoplasmic membrane to the cytoplasm where it dissociates, releasing its proton. In the cytoplasm, carvacrol attaches a potassium ion (or another ion), which will be then transported across the cytoplasmic membrane to the external environment. Once outside the cytoplasmic membrane a proton is again fixed on carvacrol, which releases the potassium ion. In its protonated (undissociated) form, carvacrol is ready to diffuse again across the cytoplasmic membrane and dissociates, releasing the proton into the cytoplasm. Adapted from Ultree *et al.*,[44] with modification

mitochondria, and β-pinene has similar effects on intact yeast cells or isolated mitochondria (Figure 2). The interest in using essential oils and their active components as modulators of cellular physiology, homeostasis and fate is due to the high diversity in the composition of essential oils in addition to the presence of a high molecular assortment in each composition. This natural and sustainable resource of diverse molecular composition and structure will open a door for targeting a wide spectrum of cellular processes. Several studies demonstrate the bioactivity of some essential oils in the treatment of certain pathophysiological conditions. Even though some essential oils may not show any bioactivity for the treatment of certain diseases, they can be used in complement to the main treatment by either lowering the appearance of side effects or the development of certain infections when the immune system has been weakened or by boosting the weakened immune and defence systems.[52,53] In the following paragraphs, two mains topics are discussed: (1) the property of essential oils to modulate the immune system and prevent apoptosis; and (2) the use of essential oils as anti-angiogenic and anti-tumoural agents.

Immunomodulatory and Anti-apoptotic Properties of Certain Essential Oils

The immunomodulatory property of certain essential oils can be illustrated by their impact on inflammation and the expression of interleukins (ILs). For example, a recent study shows that the tea tree oil (TTO) and particularly its active component, terpinen-4-ol, reduces the expression of IL-8,[52] one of the major inflammatory mediators produced during oropharyngeal candidiasis. Terpinen-4-ol affects IL-8 secretion by protein synthesis inhibition. It would be interesting to investigate, in further details, the molecular mechanism that allows terpinen-4-ol to specifically target the protein expression of IL-8. Other inflammatory processes have been shown to be inhibited by TTO and its active components. They comprise contact hypersensitivity[54] and histamine-induced oedema[55] as well as histamine-induced weal and flare reaction in human skin.[56] In addition to its immunomodulatory effects, TTO has scientifically proven antimicrobial and antifungal activities. Therefore, it could be used as a preventive treatment in order to prevent the growth of *Candida albicans* and the development of oropharyngeal candidiasis infections, especially in cancerous patients and in individuals with reduced immunity.

Other essential oils show anti-inflammatory effects such as the *Syzygium aromaticum* (Myrtaceae) or clove essential oil. In addition to its bioactive component (eugenol), this essential oil inhibits the production of cytokines (IL-1β, IL-6 and IL-10) and prevents the inflammatory action of lipopolysaccharide (LPS).[57] Bacheiga and colleagues suggest a mechanism of action for the anti-inflammatory property of eugenol, suppressing the nuclear factor kappa B (NF-κB) pathway. It is important to check these possibilities and verify at which level of NF-κB signalling eugenol is acting. A first step would be to investigate whether eugenol has a similar action as hyaluronan,[58] which may render eugenol as a natural treatment for arthritis. Moreover, since it has been shown that eugenol prevents the action of LPS, it is interesting to investigate whether eugenol acts as an antagonist binding to LPS-binding protein, and blocking the response of the Toll-like receptor (TL4) to LPS. Another possibility would be that eugenol binds to CD14. LPS acts as well in a CD14-independent manner, by directly activating the JAK/STAT (Janus-kinase/signal transducer and activator of transcription) and NF-κB pathways as well as ERK signalling, thus utilizing a rapid and direct route to affect changes in gene expression in the nucleus.[59] Therefore, it is interesting to search for the possibility of eugenol to affect the CD14-independent activation pathways induced by LPS. According to the above-mentioned results, a synergistic effect between eugenol and terpinen-4-ol on inflammatory reactions remains to be investigated.

Another mechanism of action on inflammatory responses has been observed for parsley [*Petroselinum crispum* (Apiaceae)] essential oil blocking the proliferation of phytohaemagglutinin (PHA)-stimulated splenocytes. Yet, in the case of PHA- and LPS-stimulated macrophages, nitric oxide (NO) production has been suppressed after essential oil treatment.[60] In the above-mentioned and other previous studies, the effect on cytokine production by macrophages has been suspected to occur even before LPS stimulation. This effect was significant when using neral and geranial (Table 1), the bioactive component of lemongrass [*Cymbopogon citratus* (Poaceae)] essential oil.[61] However, the activity of either lemongrass or neral and geranial showed specificity in the inhibition effect. The capacity of essential oils to act on cytokine secretion prior to mitogen stimulation of macrophages provides evidence for the preventive activity, even though it still needs to be clearly defined at the molecular level. Is there a difference between the molecular mechanism of the preventive and the therapeutic activity? Nevertheless, in addition to previous studies, Bachiega *et al.* came to the same conclusion,[62] suggesting that one of the principles of the anti-inflammatory effect of some essential oils is based on the inhibition of NF-κB activation via inhibition of IκB phosphorylation.[62] Activation or inhibition of activation of NF-κB can change the homeostatic balance of opposing cellular processes, even though the precise effect depends largely on the specific cell type and the set of genes targeted by NF-κB as well as the immediate environment. Therefore, it is necessary to investigate the result obtained with essential oil inhibiting NF-κB activation in various cell types, to search for their specificity and duration of activity. Examining essential oils and bioactive components capable of activating, in a dose-dependent manner, NF-κB by cell stimulation is also necessary, especially in neuronal cells. Recently, there has been increasing evidence for the important role of NF-κB in synaptic plasticity, learning and memory.[63] Following learning, genes with NF-κB binding sites were highly expressed.[64] Many NF-κB target genes, which are essential for synaptic plasticity and learning, include glutamate receptors (AMPA-R and NMDA-R), growth factors (BDNF, NGF), cytokines (TNF-α, TNFR), kinases (PKAc), and synaptic scaffolding proteins (PSD-95). It should be noted that if NF-κB activation is primarily protective in one location, it can be injurious in another. Thus, constitutive activation of NF-κB in some eukaryotic cells is responsible for the development of many types of tumours, since it regulates anti-apoptotic genes, especially *TRAF1* and *TRAF2*.

Some essential oils have shown anti-apoptotic properties by targeting death pathways induced by the tumour necrosis factor-α (TNF-α), with no evidence of activating the NF-κB pathway. An example is the *Mentha arvensis* (Lamiaceae) essential oil and particularly the active component, isomenthone (Figure 2), shown to inhibit TNF-α-induced cell death via the inactivation of JNK and p38 MAPK in human dermal fibroblasts.[65]

Anti-angiogenic and Anti-tumoural Potential of Essential Oils

Essential oils and their bioactive components have shown various therapeutic potential. In addition to their previously mentioned properties, they are also considered as agents with anticancer activity, with the advantage of having a mechanism of action different from chemotherapeutic agents. Several laboratories took the challenge to identify bioactive components with anticancer and anti-angiogenic properties. Monoterpenes, which have multiple pharmacological effects, were among the candidates. In a very recent study, myrtenal (Figure 2), a natural monoterpene shows an ability to suppress and restrain hepatocellular carcinoma in rats by preventing the diethylnitrosamine phenobarbital (DEN-PB)-induced upregulation of TNF-α protein expression.[66] In the essential oils of many aromatic plants such as pepper [*Piper nigrum* (Piperaceae)], cumin [*Cuminum cyminum* (Apiaceae)], mint [*Mentha piperita* (Lamiaceae)] and eucalyptus [*Eucalyptus globulus* (Myrtaceae)], myrtenal is not significantly present. However, essential oils from *Astartea* species contain the highest level of myrtenal. The development of essential oils with high percentage of myrtenal could be a promising way to use myrtenal as an anti-tumoural agent. Other terpenes showed similar effect on the growth of tumour cell lines.[67] It is obvious that the mechanism of action of these anticancer agents is based on the modulation of the apoptotic and anti-apoptotic cascades, in addition to their antioxidant activity and induction of mitochondrial stress.[68,69] In-depth analysis showed that myrtenal has excellent free radical scavenging activity and significant anticancer properties, in both *in vitro* and *in vivo* assays (in hepatoma-bearing animals).[70]

Angiogenesis is the physiological process which involves the growth of new blood vessels from pre-existing ones. Under non-pathological situation, angiogenesis is a normal and vital process in growth and development, as well as in wound healing and in granulation tissue. However, this process participates in the fundamental step of tumour transition from a dormant to a malignant state in which the tumour receives nutrients and oxygen in order to increase its growth. Therefore, many strategies for combating cancer were focused on the formation of new blood vessels near the tumour by using anti-angiogenic treatments (angiogenesis inhibitors). The search for non-toxic anti-angiogenic agents led to the investigation of the potential of essential oils as inhibitors of angiogenesis. A recent study demonstrated the capacity of *Origanum onites* (Lamiaceae) essential oil (OOEO) in the inhibition of cancer cell viability and proliferation and in the *in vitro* inhibition of tube formation and migration of rat adipose tissue endothelial cells (RATEC). The latter property indicates its capacity of blocking angiogenesis.[71] The authors showed that the cytotoxicity values of OOEO are much higher on cancer cells, which is one of the characteristics that must be considered as a standard parameter that will allow the use of essential oils in the prevention and treatment of disease. In addition, essential oils must be selective and not lead to side effects even after prolonged exposure. The presence of 64% of carvacrol in the OOEO may indicate that it is the bioactive component responsible for the anti-angiogenic and pro-apoptotic effects. It is essential to isolate the bioactive components that can inhibit angiogenesis and investigate their mechanism of action. The pro-apoptotic activity of carvacrol has been demonstrated by Liang and Lu.[72] In addition, they revealed that carvacrol is responsible for the increase of Ca^{2+} cytosolic concentration ($[Ca^{2+}]_i$) via two mechanisms. Carvacrol induced the extracellular entry of Ca^{2+} via transient receptor potential ion channels (TRPM8 and TRPV3) sensitive to protein kinase C.[73] In addition, it provoked phospholipase C-dependent Ca^{2+} release from the endoplasmic reticulum. In contrast, the same study shows that carvacrol-induced $[Ca^{2+}]_i$ rise was not responsible for cell apoptosis. However, it showed that carvacrol-induced cell death is probably activated by ROS production in glioblastoma cells. It would be interesting to look for the effect of carvacrol on Ca^{2+} signalling in neuronal cells.

Conclusion

Several active components of many essential oils are capable of modulating paradoxal responses triggered by different genes and pathways. As a consequence, understanding the mechanism of action of the bioactive components on the modulation of such paradoxical responses, within a cell or between different cell types, is a prerequisite to developing new therapeutic strategies against many different pathologies. In addition, the efforts must be concentrated to investigate the synergistic effects of the bioactive components, which allow a better and effective response. For an adequate preventive and therapeutic use of essential oils or their active components, specificity and cytotoxicity must be taken into account. It should be noted that cytotoxicity is well known[74] due to the regulations in the aroma, perfume, tobacco and cosmetic industries. While the use of essential oils in disease treatment is well studied, much more data need to be collected regarding their preventive effect. In addition, there is still a need to develop and engineer new approaches, in order to specifically address essential oil bioactive components to selected targets, which could accommodate the cytotoxicity property towards dysfunctioning loci. One of these approaches is to combine bioactive components to particles (toxins, agonists or antagonists, peptides, monoclonal antibodies etc.) that target receptors of specific benign or malignant cells. Finally, once cytotoxicity is controlled, essential oils and their components would be among the best agents to be recommended for preventive medicine, considering their high molecular diversity and wide spectrum of activity.

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