Antimicrobial activity of essential oils on pathogenic strains

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Plant products are the main innovative sources of therapeutic agents for the control and treatment of infectious diseases in humans, animals and plants. Current research on molecules and natural products focuses mainly on medicinal plants, which are selected according to their popular use. Products obtained from medicinal plants can be divided into different phytochemical categories: phenols, quinones, flavones and flavonoids, tannins, coumarins, EOs, alkaloids, lectins and polypeptides. Several studies have confirmed the antibacterial, antifungal and antiviral activity of the EO. In addition, reports showed that EO can affect biofilm formation [2, 3], producing damage to the structure of the microbial membrane. Different studies have shown that these compounds can inhibit the growth of animal pathogens. Essential oil of *Minthostachys verticillata* (Griseb.) Epling, a native medicinal plant of South America, and one of its compounds (limonene) showed antibacterial activity against bovine mastitis pathogens. Likewise, it was shown that EO was able to affect biofilm formation. Similarly, different plant essential oils were were used for the testing of the inhibitory activity against *Paenibacillus larvae*, etiological agent of American foulbrood. On the other hand, studies conducted on *Pseudomonas syringae* pv glycinea (causative agent of blight of soybean), *Xanthomonas axonopodis* (causal agent of pustule) and other phytopathogenic bacteria showed that the EO of *Coriandrum sativum, Foeniculum vulgare* and *Origanum vulgare* exhibited good inhibitory activity. Similarly, EO obtained from members of the *Lamiaceae* family (*Lavandula stoechas* ssp., *Rosmarinus officinalis*, *Origanum onites*, *Thymbra spicata*) were active against *Pseudomonas savastanoi* pv *phaseolicola* causal agent of late blight in porotos. In addition, inhibitory effects of *Thymus vulgaris* and *Origanum vulgare* EO on virulence factors of phytopathogenic *Pseudomonas syringae* strains was reported. The antimicrobial activity and low toxicity of medicinal plants are potential sources of novel antimicrobial compounds and new alternatives for the control of animal and plant pathogens.

Keywords: essential oils; medicinal plants; bacterial pathogen; antimicrobial activity; biofilm

1. Medicinal plants

From ancient civilizations, plants have been used to prepare food and medicines [1]. All cultures, throughout the world have used medicinal plants as the basis of their own medicine. This knowledge has been transmitted over time from generation to generation, serving as a basis for traditional medicine [2, 3].

Medicinal plants are distributed all over the world and it is estimated that there are more than 25.000. Plant products are the main innovative sources of therapeutic agents for the control and treatment of infectious diseases in humans, animals and plants. Current research on molecules and natural products focuses mainly on medicinal plants, which are selected according to their popular use.

Essential oils (EOs) obtained from plants are known for having antimicrobial effects. Several studies have confirmed their antibacterial, antifungal and antiviral activity [4]. Development of microbial resistance in EOs has not been described. For this reason, they have been evaluated in both animal and plant pathogens. In addition, reports showed that EO can affect biofilm formation [5, 6], producing damage to the structure of the microbial membrane [7]. As a result, EOs with antimicrobial properties, makes medicinal plants attractive from the pharmacological and commercial point of view [8].

1.1 Composition of essential oils

EOs are complex mixtures of volatile compounds, which can contain among 20-60 compounds at quite different concentrations and can be extracted from different parts of the plants. The term “essential” comes from “essence” and is related with flavors and odors produced from many plants [9] making EOs the most important raw materials for the fragance and aroma industry.

EOs are produced in the cytoplasm of plant cells and are accumulated in different parts of the plant, being able to be located in a specific plant organ: flowers, leaves, fruits and even roots or in the whole plant [10, 11]. Commonly, EOs are a combination of two to five major compounds, some of them contribute up to 70% and others are present at trace levels.

EOs compounds can be classified in two different groups according to biosynthetic origin. The major group is composed of terpenes and terpenoids, derived from the acetate-mevalonic pathway. The other group is composed of aromatic and aliphatic constituents and derives from acid and shikimic acid pathway [12]. All of these compounds are characterized by low molecular weights. The chemical composition of each EOs, depends on its provenance, according to geographical sources, and environmental factors as climate, soil, season and genetic factors. The state of development of the plant and the extraction methodology used can also affect the composition of EOs [13].

Compounds as terpenes and terpenoids are the major group of EOs including more than 15,000 compounds. They are combinations of several 5-carbon-base (C_5) units called isoprene. Monoterpenes (C_{10}) and the sesquiterpenes (C_{15}) are the principal terpenes, although hemiterpenes (C_5), diterpenes (C_{20}), triterpenes (C_{30}) and tetraterpenes (C_{40}) also are present [14]. A terpenoid is a terpene containing oxygen. The most representative molecules in terpenes group are monoterpenes (C_{10}) which constitute more than the of 70% of the EO. They may have different functional groups as carbures; alcohols; ketones, esters, aldehydes, ethers; peroxides and phenols. The first four groups are acyclic, monocyclic and bicyclic molecules and aldehydes are mostly acyclic molecules.

The aromatic compounds present in EOs are derived from phenylpropane and their presence is less frequent than terpenes. Representative families of aromatic compounds are aldehydes, alcohols, phenols, methoxy derivatives and methylene dioxy compounds.

1.2 Essential oils extraction

EOs can be extracted from the plant by different methods, among these hydro-distillation (HD), steam distillation, water and steam distillation, maceration, empyreumatic distillation, enfleurage and expression can be cited [15].

Hydro-distillation is one of the main methods where the plant material is heated up to five times its weight of water. Then, mixture of steam and oil is condensed and the oil is collected in a Clevenger type apparatus [16]. In steam-distillation EOs are extracted by direct steam formed in the still or by indirect steam formed outside and fed into the still. Then, the water-oil mixture is condensed and the oil is separated in a Clevenger type apparatus [17]. In water and steam distillation, the plant material is placed in two to three time its weight of water and it is extracted by steam produced outside the still. Again, the water-oil mixture is clatter condensed ant the oil is separated in a Clevenger type apparatus. Likewise, EOs can be extracted by maceration where plant material is immersed in a solvent of low polarity as hexane or ethyl ether. Then, EOs are separated from the extraction solvent by low pressure distillation. As the process is slow and it can take a long time, new technologies are currently being used to improve efficiency and reduce extraction time. In this way, some research groups reported on the use of ultrasound to aid in the extraction process [18].

Expression, or cold pressing, is a method mostly used for the extraction of citrus oils. It is a physical process in which the EOs glands in the peel are crushed or broken to release the oil. Enfleurage is an old process to obtain EOs, but it is hardly used today. It is a process which uses odorless fats to extract the fragrances from the plant materials. In this process, the plant parts are put in contact with the solid fat (cold enfleurage) or stirred in the melted fat (hot enfleurage) so the EOs slowly absorb into the fat [19]. Empyreumatic distillation is another old technique used to obtain essences. In empyreumatic extraction the plant material is distilled at high temperatures in the absence of air, and the oils are condensed on the walls of the closed vessel.

There are also other newer methods to obtain EOs from plants, though they are less used. In microwave-assisted solvent-extraction (MAE), microwaves are used to excite water molecules in the plant tissues causing plant cells to rupture and release the EOs trapped in the extracellular tissues of the plant [20]. The EOs of the plant are collected in the extraction solvent and may be separated later by low-pressure distillation. Ultrasound causes similar effects as do microwaves and it is used in the so-called method of Ultrasound Assisted Extraction (UAE). Another new method used to extract natural products from plants is the so-called Solvent-Free Microwave Extraction (SFME). In this method, the plant material is placed in a microwave reactor, without any added solvent or water. The internal heating of the in-situ water within the plant material distend the plant cells and leads to rupture of the glands and oleiferous receptacles. EOs are thus freed and evaporated by the in-situ water of the plant material. The evaporated EOs are then condensed and collected in a glass apparatus outside the microwave oven [21]. In the Soxhlet extraction method a low-polarity solvent (**e.g.** hexane) is used to continually extract fats and oils from a crushed plant contained in a thimble [22]. In supercritical fluid extraction (SFE) the solvent is a substance (usually CO_2) in its supercritical state, **i.e.** at a pressure and temperature beyond its critical point. A supercritical fluid is a state of matter which behaves like gases (diffuses through solids) and liquids (has the capacity to dissolve substances). Carbon dioxide is generally used because its supercritical state is somewhat easy to obtain (i.e. critical temperature 304.2 K and critical pressure 73 atm), and, more important yet, CO_2 is eliminated easily once the applied pressure and temperature are driven to normal values [23]. Finally, it has been informed in the literature that ohmic heating can also be used as a method to extract EOs from vegetables. Ohmic-assisted hydrodistillation is based on the combination of ohmic heating and distillation [24] to extract EOs from plant materials. The process is carried out by passing an alternating electrical current through materials, in which the conversion of electrical energy into heat within the material is occurred. As EOs have low electrical conductivity they do not experience direct heating, which is an advantage to preserve EO components integrity.

2. Medicinal plants for the control and treatment of bovine mastitis

Different reports on the pharmacological study of medicinal plants used in the control animal diseases are described in the literature [25, 26]. Bovine mastitis is the major disease of cattle and a cause of important financial losses to dairy industries. Different agents can cause the disease [27]. Antibiotic therapy is used commonly, but the intensive use leads to antibiotic resistance and the need to explore alternative arise. Natural products could be used as an alternative therapy for the control of this disease. Different studies on the effect of medicinal plants on bovine mastitis pathogens have been reported. Hase et al., 2013 used a topical herbal spray (AV/AMS/15) with Mastilep gel against subclinical mastitis. Results showed that the spray improve milk yield and milk fat content, and also reduced Somatic Cell Count [28]. Sunder et al., 2013 studied the effect of *Morinda citrifolia* fruit juice on bovine mastitis [29]. Diarra et al., 2013 studied the mechanism of action of cranberry extract on *S. aureus* isolated from bovine mastitis and reported that the extract has an antimicrobial activity similar of the antibiotics by altering the cell wall [30]. Sharma et al., 2014 studied an herbal preparation of *Withania somnifera, Asparagus racemosus, Emblicaofficinalis, Ocimum sanctum, Tinospora cordifolia, Tribulus terrestris*, and *Nigella sativa*. Results showed a reduction in stress before parturition, as well as an improvement in immunity in the cows tested [31]. Shafi et al., 2016 evaluated the immuno-modulatory and anti-inflammatory properties of *Ocimum sanctum* (Tulsi) against mastitis pathogens demonstrating improved activities of lactoperoxidase and myeloperoxidase as also increased phagocytic activity of milk neutrophils [32]. Reshi et al., 2017 assayed the antibacterial activity of *Fumaria indica* and *Adiantum capillus* against *Staphylococcus aureus, Escherichia coli*, *Streptococcus agalactiae* and *Klebsiella pneumoniae* subclinical mastitis isolates [33].

Different species of aromatic plants belonging to the genus *Minthostachys,* used in traditional medicine and as foods flavorings, grow in different South America countries. *Minthostachys verticillata* (Griseb.) Epling, commonly called “peperina”, stands out for its ethnomedicinal properties. The EO has biological activity, which could be attributed to the presence of monoterpenes such as pulegone, menthone and limonene, which are its main components [34, 35, 36]. An *in vitro* study determined the antibacterial activity of EO of *M. verticillata* and one of its components (limonene) against bacterial pathogens isolated from bovine mastitis. The antibacterial activity of EO and limonene was tested against commonly bovine mastitis pathogens such as *Staphylococcus aureus*, *Streptococcus uberis*, *Escherichia coli* and coagulase negative staphylococci (CNS) and then compared with antibiotics most used in bovine mastitis (cephalothin, erythromycin, penicillin, tetracycline, and oxacillin). Both EO and limonene were effective against the tested bacteria, at high concentrations. This study revealed a broad spectrum of antibacterial activity, showing a greater bacterial susceptibility to plant fractions compared to the antibiotics tested [37].

Similarly, Montironi et al., 2016, evaluated the minimum inhibitory concentration (MIC) and the minimum bactericidal concentration (MBC) of *M. verticillata* EO and limonene on fifteen *Streptococcus uberis* strains isolated of cows with mastitis from the central dairy region of Argentina [35]. Activity of vegetal compounds MICs on biofilm formation was determined. Results showed that EO and limonene were effective on seven of the 15 strains tested. MIC values for EO were 14.3 to 114.5 mg/ml (1.56-12.5% v/v), and MBC values were 114.5 and 229 mg/ml (12.5-25% v/v). On the other hand, MIC values for limonene ranged from 3.3 to 52.5 mg/ml (0.39-6.25% v/v) and MBC was 210 mg/ml (25% v/v). The MICs were evaluated by absorbance measurement (DO_{560nm}). According to these results and taking into account the values of MBC, EO showed to be more effective as antibacterial than limonene against *S. uberis* strains. As several mastitis pathogens are biofilm formers [38, 39], MICs of EO and limonene were also evaluated against S. *uberis* strains biofilm formers and both vegetal compounds reduced the biofilm production. EO MIC inhibited the biofilm formation in percentages from 88.25 to 23.50%. On the other hand, limonene MIC inhibited the biofilm formation in percentages from 92.18 to 23.20%. This study demonstrated that both EO and limonene acted as effective antibacterial against *S. uberis* strains biofilm formers.

In other study, the effect of *M. verticillata* EO and limonene on biofilm was evaluated against *Escherichia coli*, *Bacillus pumilus* and *Enterococcus faecium*. All bacteria tested formed biofilm. Both EO and limonene were able to inhibit the biofilm at different stages, during and after its formation. However, the better inhibition percentages were observed during biofilm formation (from 36.51 to 89.60% for EO and 22.06 to 89.83% for limonene) [36].

3. Medicinal plants for the control and treatment of American foulbrood

American foulbrood (AF) is a disease that causes the death of larvae and pupae of bees (Apis mellifera L.). The etiological agent is a Gram positive bacteria called *Paenibacillus larvae* [40]. The bacteria has the ability to form spores, which are resistant to adverse environmental conditions and can preserve their infective capacity for several years. AF is an infectious disease that affects honey industry throughout the world. This disease spreads very quickly through infected hives and working material.

P. larvae spores cause an intestinal infection in young larvae and reach the larval body cavity (hemocele) [40, 41]. After the hatching of the egg, the larva can be exposed to the spores. This event occur during the first 36 h [42]. The spores of *P. larvae* germinate in the lumen of the larval intestine, where the vegetative cells proliferate massively, breaking the epithelium of the midgut and invading the hemocoel, leading to a generalized infection. Then, the larva dies and becomes a viscous mass. The larval biomass is converted into bacterial biomass. When the organic nutrients decrease, the bacteria sporulate and the larvae dry in the breeding cell. Sequencing of the genome of *P. larvae* revealed its potential to produce unknown secondary metabolites, such as non-ribosomal peptides and peptide-polyketide hybrids [43].

To control the disease, oxytetracycline hydrochloride was used for many years. But resistant strains were detected. Alippi et al., 20017, reported that the tet (K) determinant of some *P. larvae* isolates can be transferred to *Bacillus subtilis,* indicating that plasmid conjugation can be implicated in the transfer of resistance to tetracycline. Therefore, thus arises the need to search for new treatment alternative [44].

The use of extracts and EOs of plants are alternative methods to control AF as a natural non-polluting alternative. Various natural products obtained from different vegetables are mentioned in the bibliography. Gonzalez et al., 2010, tested the antimicrobial activity of 10 plant species (*Achyrocline satureioides*, *Chenopodium ambrosioide*, *Eucalyptus cinerea*, *Gnaphalium gaudichaudianum*, *Lippia turbinata*, *Marrubium vulgare*, *Minthostachys verticillata*, *Origanum vulgare*, *Tagetes minuta* and *Thymus vulgaris*) against *P. larvae*. Post-hydrodistillation residual water, decoctions, and EOs of these plants were used [45]. Water remaining after hydro-distillation presented the highest antibacterial activities against *P. larvae*. However, the Eos were less active to inhibit the growth of the bacteria.

Similarly, Marghitas et al., 2011, tested the ethanolic extract of *Achillea millefolium*, *Ocimum basilicum*, *Thymus vulgaris* and *Urtica dioica* (nettle) [46]. The extracts that showed antimicrobial activity were subsequently tested antioxidant activity determined by polyphenols concentration. *Urtica dioica* presented the lowest polyphenolic content compared to the other plant extracts, although it exhibited the highest antimicrobial activity. On the other hand, *Ocimum basilicum* presented both polyphenolic content and antimicrobial activity at higher levels, whereas *Thymus vulgaris* had the lowest antimicrobial activity, presented large number of polyphenols.

4. Medicinal plants for the control and treatment of infectious diseases in plants

Different microorganisms can survive in plants as epiphytes or as pathogens. Fungi, bacteria and viruses can cause plant diseases. Bacteria can cause diseases in a wide range of plants all over the world. These bacteria, called phytopathogenic bacteria, can affect food-producing plants and affect negatively the worldwide food supply [47]. Phytopathogenic bacteria produce crops destruction and cause important economic losses.

To produce infection, bacteria must enter to plants through various types of wounds, stomata, hydathodes, nectaries or lenticels. Bacteria can survive in a film of water on a stoma, where they swim easily and can enter in the subestomatic cavity, where they reproduce. As the infection progresses, pathogens release virulence factors that allow them to advance on the host. Extracellular polysaccharides, enzymes, elicitors of the hypersensitive response, phytotoxins and growth hormones are recognized as virulence factor [48, 49, 50]. Infection symptoms are leaf spots and blotches, soft fruit rots, stored roots and organs, wilting, excessive growths, scabs and cankers [51, 52]. Different species of bacteria are associated with plant diseases. Among, them Gram negative bacteria as *Agrobacterium, Erwimia, Pseudomonas, Xanthomona* and *Xylella* are the most representative microorganisms of this group. Some Gram positive bacteria as *Clavibacter* can also cause diseases.

Antibiotics as streptomycin, tetracyclines and cycloheximide, are commonly used in the treatment and control of plant diseases. However, their intensive administration is associated to an increase in the resistance of microorganisms to antibiotics, generating undesirable effects in humans, animals and the environment. In addition, microorganisms that acquire resistance make treatments ineffective [53]. These facts have guided studies towards the search of new strategies for the prevention or treatment the diseases. Alternative control methods using compounds derived from medicinal plants can be a natural, effective, safe and inexpensive choice for the treatment of plant diseases [53]. In addition, natural compounds can reduce the release of unwanted contaminants to the environment [54].

Different studies reported the antimicrobial effect against phytopathogens and suggest that medicinal plants could be used as effective antimicrobial agents for controlling certain important agricultural phytopathogenic bacteria. EOs samples obtained from members of the Lamiaceae family (*Lavandula stoechas ssp., Rosmarinus officinalis, Origanum onites, Thymbra spicata*) were active against *Pseudomonas savastanoi pv. Phaseolicola*, causal agent of late blight [55]. Studies carried out on *P. syrinage, Xanthomonas campestris* (causal agent of pustule) and other phytopathogenic bacteria showed that the EOs of *Coriandrum sativum, Foeniculum vulgare* and *Origanum vulgare* showed good inhibitory activity [53].

Similarly, Sokmen et al., 2004 studied the antimicrobial activity of the EO of *Thymus spathulifolius*, collected in Turkey, against *Clavibacter mychiganensis, P. syringae pv. tomato* and *Xanthomonas campestris* and reported good antimicrobial activity of this oil on the three phytopathogens [56]. Paret et al., 2010 studied structural variations in *Ralstonia solanacearum* cells by micro-Raman spectroscopy after addition of EOs of palmarosa, lemongrass, and eucalyptus. Results showed that lemongrass was the EO most effective in degrading cellular components [57]. Kokoskova et al., 2011, studied EOs of species belonging to Lamiaceae family (*Origanum sp., Thymus sp., Mellisa sp., Mentha sp. and Nepeta sp*.) against pathogenic agents as *Erwiniaamylovora* and *P. syringae pv. syringae* and saprophytic agents as *Pseudomonas fluorescens, Pantoea dispersa* and *Pseudomonas agglomerans*. Results showed that EOs from *Origanum compactum, O. vulgare* and *Thymus vulgaris,* with carvacrol and thymol as the main compounds, were the most effective against all assayed bacteria [58]. Kotan et al. 2014, tested the antibacterial activity of extracts and EOs of *Turkish Achillea, Satureja* and *Thymus* species against 25 plant pathogenic agents. Hexane extracts and EOs of *Satureja spicigera* and *Thymus fallax*, composed by carvacrol and thymol, displayed antibacterial activity against the phytopathogen strains assayed. On the other hand, EOs from *Achillea* species displayed a weak antibacterial activity [59].

Several EOs were studied for their antimicrobial activity and some of them were able to inhibit *Pseudomonas aeruginosa*. Components as terpenes were also evaluated on different bacterial species obtaining good inhibitory activity [60]. In addition, mixing terpenic components with each other or mixing with antibiotics, the activity was enhanced [61, 62]. *P. syringae* is a gram-negative rod responsible of a variety of diseases in many crops, including apple, beet, beans, cabbage, cucumber, oat, olive, pea, tobacco, tomato, rice and flower species. Its control and management are difficult due to the lack of effective bactericides. *P. syringae* has been divided in many pathovars, based on the host from which it was isolated, producing symptoms such as spots, speckling and blight. Knowledge of antimicrobial activity of thyme and oregano EOs provides opportunities to test natural substances for control of phytopathogenic bacteria responsible of severe diseases that lead to economic losses in many crops [63]. Oliva et al., 2015, demonstrated the ability of *Thymus vulgaris* and *Origanum vulgare* EOs oils to inhibit the growth of *P. syringae* strains isolated from soybean. In addition, the EOs were able to inhibit the formation of biofilm and the release of phytotoxins in these strains [64]. These results have prompted further studies on the mechanism of Eos against bacterial infection.

Kim et al., 2015 screened 83 EOs for biofilm inhibition against *Pseudomonas aeruginosa and* reported that cinnamon bark oil was able to inhibit biofilm formation, as well as a decrease in pyocyanin and 2-heptyl-3-hydroxy-4(1H)-quinolone was detected. In addition, swarming motility and hemolytic activity was affected [65]. Rim Song et al. 2016, tested the antibacterial activity of EOs from 49 plant species against *P. syringae*. EOs from *Pimenta racemosa, Pimenta dioica, Melaleuca linariifolia, Melaleuca cajuputii,* and *Cinnamomum cassia* inhibited *P. syringae* strains [66].

One of the biggest challenges of the food industry is to reduce the use of antibiotics in food-producing animals and plants, focusing the research studies on the search for alternative control methods. The antimicrobial activity and low toxicity of medicinal plants are potential sources of novel antimicrobial compounds and new alternatives for the control of animal and plant pathogens. Consequently, the preservation of medicinal plant species is vital [13, 63, 64].

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