UNIVERSITÀ DEGLI STUDI DI MILANO

SCUOLA DI DOTTORATO
Scienze molecolari e biotecnologie agrarie, alimentari ed ambientali

DIPARTIMENTO
Scienze Molecolari Agroalimentari

CORSO DI DOTTORATO
Chimica, Biochimica ed Ecologia degli Antiparassitari
XXIV Ciclo

TESI DI DOTTORATO DI RICERCA

TRP ACTIVE COMPOUNDS FROM FOOD PLANTS AND THEIR PROPERTIES AS ANTIMICROBIAL AND BIOCIDES

(AGR/12)

DOTTORANDA:

CATERINA CALAMELLO
Matr. n. R08189

TUTORS: Prof.ssa ANGELA BASSOLI

Prof.ssa PAOLA SARDI

COORDINATORE: Prof. DANIELE DAFFONCHIO

Anno Accademico 2010-2011

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To my beloved family

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1 INTRODUCTION

1. 1 Natural Products in Crop Protection

The success of modern agricultural practices is due in part to discovery and adoption of chemicals for pest control. Indeed, the tremendous increase in crop yields associated with the "green" revolution would not be achieved without the contribution of these syntheyic compounds. The abundance of high quality food in developed nations has all but eliminated concerns about access to food in these countries. However, concerns over the potential impact of pesticides on the environment has now become more pressing and more stringest pesticide registration procedures such as the Food Quality Protection Act in the United States have been introduced (House Resolution -1627, 1996).

These new regulations have reduced the number of synthetic pesticides available in agriculture. Therefore, the current paradigm of relying almost exclusively on chemicals for pest control may need to be reconsidered (Dayan *et al*., 2009).

New pesticides, including natural product-based pesticides are being discovered and developed to replace the compounds lost due to the new registration requirements. New pesticides are also needed to combat the evolution of resistance to pesticides (Copping *et al*., 2007).

Dyan *et al*., (2009) considered the historical use of natural products in agricultural practices, the impact of natural products on the development of new pesticides, and the future prospects for natural products-based pest management. However, they stated that many natural compounds have been descovered and patented, but are not commercially available for numerous reasons.

Following, are reported some worldwide estimated values on agents damaging agricultural crops, losses occurring in field and during fruit and vegetable transport and storage and use of pesticides in the world (Peshin *et al*., 2009).

- Number of pest species damaging agricultural crops: 9.000 insects and mites, 50.000 pathogens, 8.000 weeds. In total ~ 70.000 different species.
- Range of crop production destroyed by pests in field:

35-42% (14% by insects, 13% by pathogens, 13% by weeds). These losses have been calculated, despite the early investment of about $ 40 billion for the application of 3 million metric tons of pesticides, worldwide (Table 1.1). Anyway, the value of this crop loss is estimated to be $ 2.000 billion per year, yet there is still about a 4 dollar return per dollar invested in pesticide control.

- Losses that occur during transport and storage of plant crops.
  Woldwide, an estimate of 25% food losses occur during transport and storage due to bacteria, fungi, insects, rodents and birds. In the US, post harvest food losses to pests are estimated to be about 10%. Thus, despite all pesticide use and other non-chemical pest controls, we are losing from 50% to 60% of all potential food production to pests worldwide.

Table 1.1 Annual estimated pesticide use in the world (Peshin *et al*., 2009).

Country/region	***Pesticide use 10^6 metric tons***
United states	0.5
Canada	0.2
Europe	1.0
Other developed	0.5
China	0.2
Asia, developing	0.3
Latin America	0.2
Africa	0.1
Total	**3.0**

1.1.1 Crop protection, a historical overview

The history of the development of farming is strictly related to the parallel evolution of the pest control strategies. Insects and other herbivores reduced yield by eating the crops; pathogens attacked the crops, and weeds competed with primary crops and range forage, often giving rise to periods of famine or causing serious food poisoning in vast part of the world population. Various chemical pest control strategies have been tried over the centuries and here it's shortly reported the changes in crop protection during the last centuries. The period before the eighteenth century, growers of crops had to fight insects, diseases and weeds with inadequate tools. The growers lost a considerable proportion of

crops to pests during productions, storage and distribution. Prior to the 1950's crop protection tools included mechanical removal of weeds, a few number of synthetic organic chemicals and inorganic materials. They were non-selective, persistent and toxic to many forms of life. Herbicides included ashes and salts. Insecticides included arsenic and fluoride. Fungicides included copper, ash and sulphur.

During the eighteenth century insecticides included botanical compounds as nicotine, rotenone and pyrethrums. These products were more specific in terms of control, but not very stable for use in agriculture given rapid breakdown in the environment.

Sulfur is the oldest effective fungicide and is still in use today. Sulfur and the copper containing Bordeaux mixture (1885) were the major pesticides in use in vines to control powdery and downy mildew and specific weeds until the advent of synthetic organic compounds in the 1940's.

The period between the early 1900's and post Second World War saw the increased reliance on chemical means to control pests and diseases. Many pesticides were the by-products of coal gas production or other industrial processes. Early organics as nitrophenols, chlorophenols, naphthalene and petroleum oils were used for insect and pest control. Most of the products used were non-selective and toxic to both users and non-target organisms. Herbicides for the era included ammonium sulphate and sodium arsenate, used extensively on fruits and vegetables, with the result of accumulation of harmful residues.

The modern era of synthetic organic pesticides began in the 1930's. Medical and military researches produced the discovery of many pesticide families that are still in use today. A real breakthrough in weed control occurred with the introduction of phenoxy herbicides, in particular 2,4-dichlorophenoxyaceti acid (2,4-D), in the 1940's, for broad-spectrum broadleaf weed control in corn and cereal crops.

The early twentieth century brought the introduction of organomercurials for disease control and organochlorines such as DDT for insect control. These products were very persistent and efficient with a wide spectrum of action: good properties for agriculture and for public health, but, as was understood in the following years, not desirable after control was achieved. The introduction of organophosphates brought a new class of insecticides with reduced persistence and lower risks to the environment. Originally discovered as nerve poisons, these chemicals were highly toxic, had a broad spectrum of activity against insects and showed only moderate stability in soil, on crops and the environment. The latter was a positive characteristic from an environmental and human safety view, but it

also resulted in the need to make several applications over a growing season, increasing the potential for exposure to human and wildlife and the development of insect resistance. Another negative characteristic was their high acute toxicity, which caused hazards to applicators, and field workers. A number of organophosphates have been restricted or eliminated.

Carbamates also came into use in the 1950's and are still used today. These chemicals have relatively low mammalian toxicity and are selective in that they are toxic to target insect pest but not most helpful insects.

Another key innovation during this period was the manufacture and refinement of equipments for effectively applying these materials to crops and plants.

The post-war period, from 1950's to 1970's, saw the rapid introduction of a range of new pest control tools including many insecticides and fungicides still in use. The evolution of materials continued with new chemical families discovered that offered reduced persistence and environmental concerns along with attractive and valued benefits to producers and end-users.

This period saw the introduction of soil residual herbicides such as the triazine herbicide atrazine, evolving to the introduction of non-residual products like glyphosate in the 1970's. Several broad-spectrum fungicide families with active ingredients, like chlorothalonil, were introduced followed by the introduction of highly selective systemic fungicides, that worked on specific metabolic processes in specific pathogens. It was also the period of introduction for synthetic pyrethroid insecticides. The discovery and use of systemic and single mode of activity pesticides also created resistance concerns and the introduction of resistance management strategies to keep in use these products in a long time.

The evolution of scientific procedures for evaluating the impact of pesticides on users and the environment along with the introduction of newer classes of reduced risk products led to the removal of many older classes of chemistries that were persistent with negative impacts on the environment in front of which peoples became more sensitive. A classical example is the path of DDT: over the years, it became clear that this organic material had adverse impacts on the environment, leading to reduced populations of birds and some aquatic organism. One of its benefits, that of long residual life, was also a negative aspect. This insecticide had very long half-lives and became widely distributed in nature by accumulation through the food chain and the atmospheric distribution, so most of the organchlorine insecticide were banned in the 1970's.

At the end of the twentieth century there was the evolution to newer classes of highly specific, low toxicity and low use rate, insecticides and fungicides. These products were used at the rates of millilitres or grams per hectare. Insecticides that controlled only certain stages of the lifecycle without harming beneficial species in crops were registered. Likewise fungicides were introduced that featured both forward and backward systemic activity to control diseases. Today, we see the registration of low use rate herbicides that interact with specific biochemical sites to produce selective and environmentally safer weed control.

This period also saw the refinement of products in terms of use patterns with the introduction of newer and more user-friendly and environmentally safe formulations.

As well, we entered into an era of genetically engineered pesticides and crops designed to reduce or eliminate the use of pesticides in controlling specific pests. The use of pheromones to disrupt insect mating habits and the use of microorganisms to combat diseases were also introduced and used on a broader scale in specific crops like greenhouse vegetables or fruit crops.

The latter half of the 1990's saw the introduction of herbicide tolerant crops including soybean, corn and cotton using both traditional and transgenic breeding techniques, and the introduction of varieties of corn and cotton resistant to corn borer and boll weevil respectively with the reduction of the volumes of pesticides used on crops.

In this period not only the tools but also the approach to fight the pests was changing: it became holistic and more rational, more careful for the environment and human health and also economic savings for the farmer. It's the Integrated Pest Management (IPM) era (Ehler, 2006).

IPM increases the sustainability of farming systems and it's recognized that the farmer should manage pathosystems rather than control pathogens and crop protection became a matter of searching for the optimal combination of different control measures.

The aim is not the eradication of pest but keeping their populations at acceptable levels. Non-chemical means of crop protection such as biological control and rotation schemes are developed and improved. Regular monitoring of pests and their natural enemies and antagonists are important to detect economic or treatment thresholds for the rational application of pesticides. How we can see from the summary of the pest control history there was, along the years, two shifts of focus. In the second half of 20^{th} century, attention shifted from the pathogen to the pathosystem, and at present we are witnessing a further shift to a focus on the whole production system. So, crop protection is now seen as just

one activity among many in agricultural production systems and improvement of crop protection is no longer seen as separate from goals such as maximizing yield and minimizing inputs.

Herbicides, insecticides and fungicides followed a parallel development over the same 50 year period, namely, introduction of new, effective synthetic organic beginning in the 1940's, banning or restricted in the 1970's to the present and development of less risky alternative that continue in widespread use (Wheeler, 2002).

Intelligent use and a basic understanding of pesticides are key factors to their safe use and continued availability. The public perception of risk, which has a strong influence on pesticide availability and the research that undergoes it, can be improved through better communications between the scientific community and the public.

1.1.2 Problems and limitations of agrochemicals

Initially, the benefits these new chemicals brought to agricultural production were thought to be without major disadvantages; however, ecological and human health risks and the economic costs of heavy, widespread use of broad-spectrum chemical pesticides are becoming more apparent. In addition to the potential for adverse effects on human and environmental health, there are growing concerns about the durability of current approaches to pest management. The disruption of inherent natural and biological processes of pest management, the resistance to pesticides developed by many major pests, and the frequency of pesticide-induced or-exacerbated pest problems suggest that dependence on pesticides as the dominant means of controlling pests is not a durable solution. The failure to develop economically viable pesticides for some of the most damaging pests and the economic costs of continual pesticide application has also led to an interest in alternative approaches to crop protection.

Some new approaches to pest control have been adopted and good potentialities have been found in: increase of plant resistance with traditional breeding or through innovative biotecnology or chemical compounds; biological control with use of microbiological products; find of new active principles using innovative chemical methods or natural compounds as leads (Müller, 2002; Stetter *et al.*, 2000; Ragsdale, 1999).

Biological control methods are interesting tools to control the diffusion and the growth of parasites and pathogens. The control of harmful organisms could happen in several ways:

with the emission in the environmental of antibiotic secondary metabolites, with phenomena of parasitism or predation or with competitive organisms that fast colonize the media and make it inhospitable. Microbial pesticides have been used in agriculture for many years and the most widely used are subspecies strains of *Bacillus thuringensis* or Bt - a classical example by now-. They exhibit several advantages over synthetic chemicals that include safety for nontarget organisms, tendency to biodegrade, low cost to develop, and good compatibility with IPM programs. Drawbacks that might suggest further areas of research include limited product shelf life and effective life in the field, moreover they require much more knowledge for growers to use them with the same efficacy as with conventional pesticides.

The development of resistant plants to adverse conditions (biotic or abiotic) has been the result of natural selection, breeding programs and, more recently, of seed engineered that contain a gene for insect control or herbicide resistance.

Natural products are one of the most fertile sources of novel chemistries. Metabolites extracted from different organisms (plants, animals, fungi or prokaryotic) are an important sources of pesticide compounds *per se* or as leads for chemical synthesis. The history of plant protection shows some important examples of this kind of compounds in classes of fungicides, herbicides and insecticides.

1.1.3 Agrochemicals from natural compounds

The concept of 'natural pesticides' arose early in the development of agriculture. Indeed, the Lithica poem (c. 400 B.C.) states 'All the pests that out of earth arise, the earth itself the antidote supplies'. Greek and Roman scholars such as Theophrastus (371–287 B.C.), Cato the Censor (234–149 B.C.), Varro (116–27 B.C.), Vergil (70–19 B.C.), Columella (4–70 A.D.) and Pliny the elder (23–79 A.D.) published treaties on agricultural practices to minimize the negative effects of pests on crops. Methods such as mulching and burning, as well as the use of oils for pest control were mentioned. Chinese literature (ca. 300 A.D.) describes an elaborate system of biological control of caterpillar infestations in citrus orchards. Colonies of the predatory ants (*Oecophylla smaragdina*) were introduced in citrus groves, and bridges made of bamboo allowed the ants to move between trees. A survey of the Shengnong Ben Tsao Jing era (25–220 A.D.) shows that 267 plant species were known to have pesticidal activity. Finally, the use of beneficial insects to control other insect pests was mentioned by Linnaeus as early as 1752, and he won a prize in 1763

for an essay describing the biological control of caterpillars.

The European agricultural revolution that followed in the 19th century was accompanied by more extensive and international trade that resulted in the discovery of botanical insecticidal powders from *Chrysanthemum* flower heads and *Derris* root which contain pyrethrum and rotenone, respectively.

The utilization of natural products in pest control may be considered from a number of standpoints. First, the variety of structural types provides a rich source of compounds models for conventional screening programs. Second, consideration of the known biological activity of a natural product may lead to its application in pest management, either directly or after structural modification (Hedin *et al*., 1985). Furthermore, since these products are almost exclusively derived from pathways associated with secondary metabolism, these compounds have a high likelihood to possess some biological activity against other organisms, often via novel mechanisms of action, which is particularly important since new modes of action are so deeply needed as pests continue to evolve resistance to the compounds currently available. An important benefit of natural product-based pesticides is their relatively short environmental half-lives, which is due to the fact that they do not possess 'unnatural' ring structures and contain relatively few halogen substituents (Dayan *et al*., 2009).

1.1.3.1 Natural products for plant pathogen management

Many natural compounds and preparations have been described with activity against bacterial or fungal plant pathogens. Indeed, plants protect themselves from microbial attacks with both constitutive antimicrobials and compounds induced by the attacking pathogen (phytoalexins). Phytoalexins have not been directly exploited as fungicides, but natural products have been used to indirectly protect plants from pathogens by induction of systemic acquired resistance (SAR), including phytoalexins. These SAR-inducing compounds and preparations are termed elicitors. Since such activity is indirect, the pathogen cannot evolve resistance directly to the elicitor, making such products excellent candidates for integrated disease management. Elicitors are generally not as effective as good chemical fungicides, partly because the timing of elicitor application and threat to the crop by a pathogen is crucial, but difficult to maximize. A range of microbially-derived products are also available for management of plant diseases.

Plant essential oils

Essential oils are volatile, natural, complex compounds characterized by a strong odour and are formed by aromatic plants as secondary metabolites. They are usually obtained by steam or hydro-distillation first developed in the Middle Ages by Arabs. In nature, essential oils play an important role in the protection of the plants as antibacterials, antivirals, antifungals, insecticides and also against herbivores by reducing their appetite for such plants. They also may attract some insects to favour the dispersion of pollens and seeds, or repel undesirable others. Essential oils are liquid, volatile, limpid and rarely coloured, lipid soluble and soluble in organic solvents with a generally lower density than that of water. They can be synthesized by all plant organs, i.e. buds, flowers, leaves, stems, twigs, seeds, fruits, roots, wood or bark, and are stored in secretory cells, cavities, canals, epidermic cells or glandular trichomes. Essential oils are very complex natural mixtures which can contain about 20–60 components at quite different concentrations. They are characterized by two or three major components at fairly high concentrations (20–70%) compared to others components present in trace amounts. Generally, these major components determine the biological properties of the essential oils. The components include two groups of distinct biosynthetical origin. The main group is composed of terpenes and terpenoids and the other of aromatic and aliphatic constituents, all characterized by low molecular weight. Due to their bactericidal and fungicidal properties, pharmaceutical and food uses are more and more widespread as alternatives to synthetic chemical products to protect the ecological equilibrium (Bakkali *et al*., 2008). Several plant essential oils are marketed as fungicides to farmers. These include jojoba (*Simmondsia californica*) oil, rosemary (*Rosmarinus officinalis*) oil, thyme (*Thymus vulgaris*) oil, clarified hydrophobic extract of neem (*Azadirachta indica*) oil, and cottonseed (*Gossypium hirsutum*) oil with garlic (*Allium sativum*) extract (Dayan *et al*., 2009).

Extract of giant knotweed

An extract of the giant knotweed (*Reynourtria sachalinensis*) is used in Europe for the control of a wide spectrum of both fungal and bacterial plant diseases. It is especially effective against powdery mildews and is used primarily on glasshouse and ornamental plants. It apparently acts indirectly by induction of plant defenses (Daayaf *et al*., 1997; Carlin *et al*., 2004). Down-regulating chalcone synthase, a key enzyme of the flavonoid

pathway, resulted in the nearly complete suppression of induced resistance by this product (Fofana *et al*., 2005). The main active elicitor compound(s) of this preparation are physcion and emodin, a known antimicrobial compound. While most of the activity seems to be associated with physcion, the photodynamic compound emodin can also generate reactive oxygen species in the presence of sunlight. Therefore, emodin-dependent oxidative stress may also induce SAR to plant pathogens.

Antibiotics from actinomycetes

A relatively large number of fermentation secondary products from actinomycetes, mostly *Streptomyces* spp., are fungicidal. Some of them have been commercialized and used extensively as agricultural fungicides in Japan, and to a lesser extent in other parts of the world. Since these compounds are considered antibiotics, they are not accepted for organic farming in the U.S., except for streptomycin for fire blight control in apples and pears. This is paradoxical since streptomycin is an important pharmaceutical and none of the others mentioned below are used as human pharmaceuticals. Blasticidin-S (Figure 1.1) from the soil actinomycete, *Streptomyces griseochromogenes* is used as a curative treatment against rice blast disease in eastern Asia (Kimura *et al*., 1996). It inhibits protein synthesis in target pathogens. Some blasticidin-S-resistant microbes detoxify the fungicide by deamination. It is active on a wide range of pathogens, but can cause damage to some crops. Kasugamycin (Figure 1.1) from *Streptomyces kasugaensis* has been used for rice blast and other crop diseases in Japan. It interferes with tRNA/ribosome interactions and inhibits protein synthesis. Mildiomycin (Figure 1.1) from the soil actinomycete *Streptoverticillium rimofaciens* is used primarily in Japan for control of powdery mildews. Its mode of action is thought to be inhibition of protein synthesis by targeting peptidyl-transferase (Om *et al*., 1979). Natamycin (Figure 1.1) from *Streptomyces chattanoogensis* is used primarily on ornamentals. It has a novel mode of action by binding ergosterol, an integral component of fungal cell membranes, thereby causing membrane dysfunction. *Streptomyces rimosus* produces oxytetracycline (Figure 1.1) that is used for control of bacterial diseases. Again, it inhibits protein synthesis by disrupting t-RNA/ribosome interactions and has pharmaceutical uses. The polyoxins (polyoxin B and polyoxorim, Figure 1.1) from *Streptomyces cacoai* are also used as agricultural fungicides (Isono *et al*., 1979). These compounds may act through inhibition of fungal cell wall biosynthesis. In

addition to being used as a pharmaceutical, streptomycin (Figure 1.1) (from *Streptomyces griseus*) is used for bacterial plant diseases. It acts by interference with prokaryotic protein synthesis by binding the 30S ribosomal subunit. Finally, validamycin (Figure 1.1) from *S. hygroscopicus* is used for *Rhizoctonia* spp. control on a variety of crops. It inhibits trehalase (Kameda *et al*., 1987), an enzyme necessary to fungi for generation of glucose to growing hyphal tips. Knocking out this enzyme stops growth, so the compound is essentially fungistatic. In Europe the use of antibiotics in agriculture is forbidden; in Italy it was banned in 1972.

Chitin components

Chitin (N-acetylchitosan) and chitosan (poly-D-glucosamine) are found in fungal cell walls and arthropod exoskeletons. Chitosan is an effective elicitor of SAR to pathogens, including phytoalexin synthesis, in plants (Hahn, 1996).

Presumably, plants have evolved a receptor/signally system to sense fungal pathogens in order to initiate chemical warfare with them. Preparations of chitin/chitosan from both crustacean exoskeletons and dried yeast (*Saccharomyces cerevisisae*) hyrdrolysate are sold as fungicides. How much of the fungicidal effect of chitosan is due to induction of resistance mechanisms of the crop is difficult to separate from possible direct fungicidal effects as chitosan possess some direct fungicidal activity (Hirano *et al*., 1989).

Cinnamaldehyde

Cinnamaldehyde (Figure 1.1) is found in several plants, but seeds of the weed *Cassia obtusifolia* are especially rich in it. It is usually synthesized chemically for use as an agricultural fungicide on a variety of crops. Its mode of action is apparently through inhibition of synthesis of the fungal cell wall component chitin (Bang *et al*., 2000; Kang *et al*., 2007). The substance has low mammalian toxicity, although it can cause moderate eye and skin irritation. Cinnamaldehyde is not soluble in water and is degraded rapidly in the soil, and it is not expected to pose any hazard to non-target organisms or to the environment (Copping *et al*., 2007).

Harpin proteins

The plant pathogen *Erwinia amylovora* that causes fire blight in apples and pears, produces a 40 kD protein termed 'harpin protein' that induces SAR in plants (Wei *et al*., 1992; Dong *et al*., 1999). It is produced by heterologous expression of the gene for this protein from *E. amylovora* in *Escherichia coli*. Since it induces SAR, it decreases susceptibility to a broad range of fungal, bacterial, and viral diseases, as well as to nematodes.

Laminarine

This product is a preparation of the storage polysaccharide (a β-1,3-glucan with some β-1,6-linked branches) of the brown alga *Laminaria digitata*. (Klarzynski *et al*., 2002). Laminarine has no antifungal activity in its own right, but it stimulates the plant's natural defence mechanism, rendering it much less susceptible to attack; it acts as a systemic acquired resistance (SAR) inducer. It is recommended for use in cereals, particularly wheat (Copping *et al*., 2007).

Extract of *Macleaya cordata*

An extract of the plant *M. cordata* is sold as a fungicide. Its greenhouse activity is comparable to synthetic fungicides (Newman *et al*., 1999). The preparation contains numerous alkaloids, but it may be acting through induction of SAR.

Strobilurins

Strobilurin and the related antifungal oudemansin (Figure 1.1) are produced by basidiomycetes that colonize dead wood. These compounds, which provide an advantage over competing fungi, have served as lead structures for commercialized synthetic analogs such as azoxystrobin and kresoxym-methyl (Figure 1.1). These compounds inhibit mitochondrial respiration by blocking the ubiquinone receptor (Kraiczy *et al*., 1996). Resistance to this class of fungicides has already evolved (Ishii *et al*., 2001).

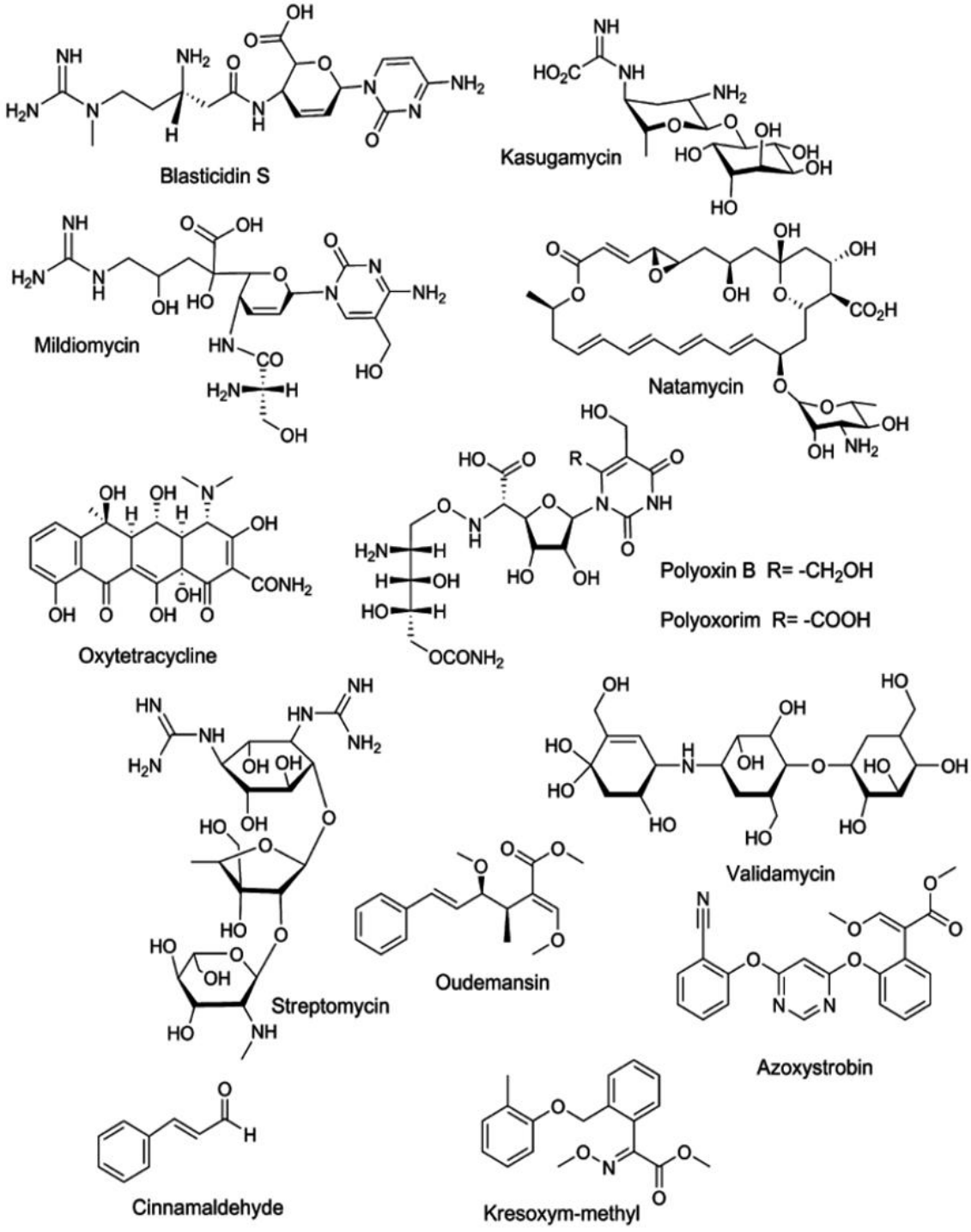


Figure 1.1 Structures of the natural fungicides and bactericides mentioned in the text (Dayan *et al.*, 2009).

1.1.3.2 Natural products for insect management

Recent reports indicate that the use of natural product and natural product-derived insecticides continue to increase, whereas sales of organophosphates are declining. Indeed, three out of the five most commonly used insecticides classes (neonicotinoids, pyrethroids, and other natural products) are natural product or natural product-derived.

Neem-based products

The seeds from the Indian neem tree, *Azadirachta indica*, are the source of two types of neem-derived botanical insecticides; neem oil and medium polarity extracts. Neem seeds contain numerous azadirachtin analogs (Figure 1.2), but the major form is the tetranortriterpenoid, azadirachtin or azadirachtin A, and the remaining minor analogs likely contribute little to the overall efficacy of the extracts (Isman, 2006). Azadirachtin is well known as a potent antifeedant to many insects. At the physiological level, azadirachtin blocks the synthesis and release of molting hormones (ecdysteroids) from the prothoracic gland, leading to incomplete ecdysis in immature insects. In adult female insects, a similar mechanism of action leads to sterility (Isman, 2006).

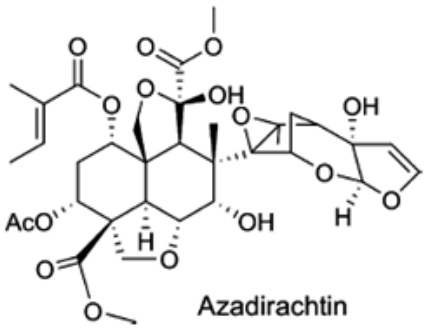


Figure 1.2 Structure of the major bioactive constituent in Neem (Dayan et *al.*, 2009).

Spinosads

Spinosad (Figure 1.3) is a mixture of spinosyn A and spinosyn D, originally isolated from the soil actinomycete, *Saccharopolyspora spinosa*. Spinosad is recommended for the control of a very wide range of caterpillars, leaf miners, thrips and foliage-feeding beetles. Spinosyns have a novel mode of action, primarily targeting binding sites on nicotinic

acetylcholine receptors that are distinct from those at which other insecticides exert their activity, leading to disruption of acetylcholine neurotransmission. Spinosad also has secondary effects on c-amino-butyric acid neurotransmission. The result of this mode of action is hyperexcitation and disruption of an insect's nervous system (Sparks *et al*., 2001). Spinosad may also be used on row crops (including cotton), vegetables, fruit trees, turf, vines and ornamentals (Porteus *et al*., 1996; Salgado *et al*., 1997).

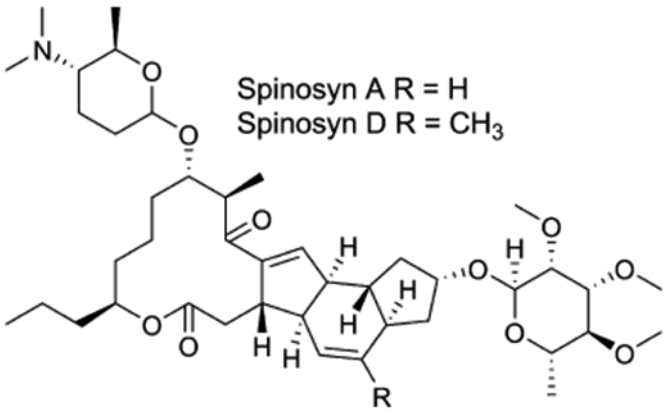


Figure 1.3 Structure of the major bioactive constituents isolated from the soil actinomycete, *Saccharopolyspora spinosa* (Dayan *et al*., 2009).

Pyrethrum

Pyrethrum refers to the oleoresin extracted from the dried flowers of *Tanacetum cinerariaefolium* (Asteraceae) and is the source of the pyrethrins, chrysanthemates and pyrethrates (Copping *et al*., 2007; Isman 2006). Among the natural pyrethrins, those incorporating the alcohol pyrethrolone, namely pyrethrins I and II (Figure 1.4), are the most abundant and account for most of the insecticidal activity. The symptoms of pyrethrin poisoning are characterized by hyperexcitation, convulsions, seizures, and finally followed by death. These symptoms are a result of the neurotoxic action, which block voltage-gated sodium channels in nerve axons. Unfortunately, the pyrethrins are extremely unstable when exposed to air and ultraviolet light; however, they are recommended for control of a wide range of insects and mites on fruit, vegetables, field crops, ornamentals, glasshouse crops and house plants, as well as in public health, stored products, animal houses and on domestic and farm animals. Pyrethrum is approved for use as a broad-spectrum organic insecticide under many trade names.

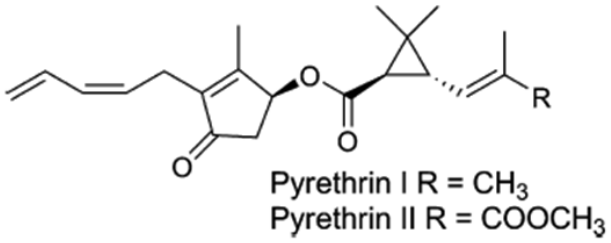


Figure 1.4 Structure of the major constituents of pyrethrum (Dayan *et al*., 2009).

Rotenone insecticides

Rotenone has been used as an insecticide for over a century and its use as a fish poison dates back even further (Isman, 2006). Typically, products containing rotenone are preparations from plant of the genus *Derris* or *Lonchocarpus* (Leguminosae). The principal commercial form of the botanical insecticide rotenone comes from Cubè resin, a root extract of *Lonchocarpus utilis* and *Lonchocarpus urucu*. Although rotenone is the primary major constituent in products containing these preparations, a second isoflavone, deguelin, also contributes significantly to the activity (Caboni *et al*., 2004; Fang *et al*., 1999) (Figure 1.5). Rotenone blocks respiration by inhibition of electron transport at the complex I (Hollingworth *et al*., 1999; Chauvin *et al*., 2001).

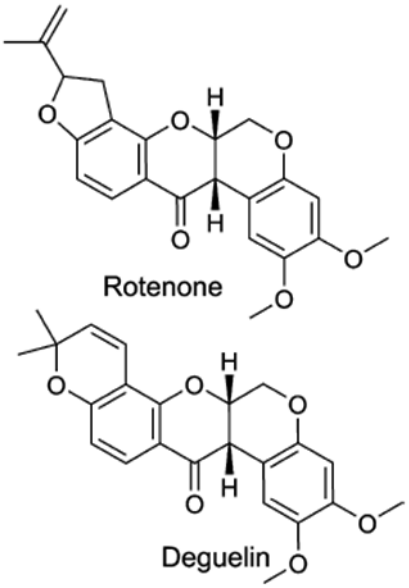


Figure 1.5 Structures of rotenone and deguelin; major constituents in rotenone insecticides (Dayan *et al*., 2009).

Avermectins and milbemycins

The structurally similar avermectins and milbemycins, both discovered from *Streptomyces* spp. culture broths, have had huge impacts in the field of animal health as agents against worms, ticks and flies (Campbell, 1989; Kornis, 1995). The impact as crop protection insecticides has been less dramatic but nevertheless significant. Abamectin, a natural fermentation product of *Streptomyces avermitilis* contains > 80% avermectin B1a and <20% avermectin B1b (Hayes *et al*., 1990). The avermectins are both insecticides and acaricides which are effective by either contact or ingestion. The target for avermectins is the GABA receptor in the peripheral nervous system. Avermectins stimulate the release of GABA from nerve endings and enhance the binding of GABA on the post-junction membrane of muscle cells of insects and other arthropods. This eventually results in an increased flow of chloride ions into the cell, with consequent hyperpolarisation and elimination of signal transduction, resulting in an inhibition of neurotransmission (Jansson *et al*., 1996). Avermectins are not registered as organic insecticides. Milbemectin is derived from the soil bacterium *Streptomyces hygroscopicus* subsp. *aureolacrimosus*, and used for mites and some insects control (Mishima, 1983). Milbemycin has the same mode of action as that of avermectins in that they potentiate glutamate and GABA gated chloride-channel opening (Arena *et al*., 1995). No organic insecticides containing milbemycins have been commercialized.

***Ryania speciosa* preparations**

Having been in use for more than half a century, *Ryania* is an insecticide obtained from the roots and stems of a South American shrub (*R. speciosa*). *Ryania* consists of powdered parts of *R. speciosa* that contains up 0.16–0.2% of the bioactive ryanodine, a complex polycyclic, polyhydroxylic diterpene. Ryanodine is effective by either contact or ingestion. Ryanodine and related alkaloids affect muscles by binding to the calcium channels in the sarcoplasmic reticulum. This causes calcium ion flow into the cells, and death follows very rapidly (Casida *et al*., 1995). *Ryania* has relatively low toxicity to mammals.

Sabadilla

Sabadilla is derived from the seeds of plants from the genus *Schoenocaulon* and is predominantly from the sabadilla lily (*Schoenocaulon officinale*). Sabadilla has been used

as an insecticide for many years by native people of South and Central America. The activity of sabadilla preparations has been attributed to the alkaloids cevadine and veratridine which typically exist in a 2:1 ratio and are collectively referred to as veratrine (Barton *et al*., 1954). Veratrine alkaloids from sabadilla have a mode of action that is similar to that of the pyrethrins. They are non-systemic insecticides with contact action. Initial effects include paralysis, with death occurring later (Copping *et al*., 2007; Crosby, 1971; Catterall, 1980). Sabadilla is considered among the least toxic of botanical insecticides, with an oral LD50 of 4000–5000 mg/kg. Sabadilla is effective by either contact or ingestion and has been effective against caterpillars, leaf hoppers, thrips, stink bugs and squash bugs.

Nicotine

Aqueous tobacco (*Nicotiana tabacum*, *N. glauca* or *N. rustica*) extracts containing the alkaloid nicotine have long been used to control crop insect pests (Schmelz, 1971). Nicotine exerts its insecticidal effect by mimicking acetylcholine and interacting with nicotinic acetylcholine receptors (nAChRs), a major excitatory neurotransmitter in the insect CNS (Yamamoto, 1998).
After acetylcholine is released by the presynaptic cell, it binds to the postsynaptic nicotinic acetylcholine receptor and activates an intrinsic cation channel (Ujaóry, 1999; Brossi *et al*., 1998). Unfortunately, nicotine is highly toxic to mammals and extreme care must be used since it is readily absorbed through the skin.

1.1.3.3 Natural products for weed management

The management of weeds has been a major problem since the inception of agriculture. Manual labor in ancestral farming practice is expended mostly on hand weeding of fields. Today, herbicides account for more than half of the volume of all agricultural pesticides applied in the developed world and the public has expressed concern about the potential health and environmental impact of these compounds. Partly due to this, organic agriculture has received a recent surge in popularity. Organic agriculture does not allow synthetic pesticides, including herbicides. Weed management under organic agriculture practices is very problematic. While most methods rely on soil cultivation, hand hoeing,

biocontrol, organic mulches, and ironically plastic (synthetic) ground cover, and the use of some natural products is permitted.

Corn gluten meal

Corn (*Zea mays*, L.) gluten meal is a byproduct of corn milling. It is commercialized as both a fertilizer and a pre-emergence herbicide on lawns and high-value crops (Quarles, 1999; Christians, 1990). Corn gluten has no effect on existing weeds, but it has a broad-spectrum of activity on the germination and development of young emerging plants (Gough *et al.*, 1999; Liu *et al.*, 1997). Hydrolysis of corn gluten by soil microbes releases several phytotoxic dipeptides and a phytotoxic pentapeptide (Figure 1.6). The exact mode of action of these oligopeptides is not known but they affect cell wall formation, membrane integrity, and nuclear development. Corn gluten may be considered a slow-release proherbicide since it must be hydrolyzed to release the active ingredients.

Acetic acid

Acetic acid has been used as a weed control agent for several centuries. Diluted aqueous solutions of up to 20% acetic acid are now sold as horticultural vinegar, or in mixtures with other natural products, for non-selective weed management. Acetic acid is a burn down, non-selective herbicide. Therefore, it is used for non-cropland areas, such as railway rights-of-way, golf courses, open space, driveways and industrial sites. Acid acetic solution (10-20%) provide greater than 80% control of the most small weeds. As is common with burn down herbicides, acetic acid kills the aerial portions of plants, but does not control the underground parts, and plants typically reemerge from the root system after a few days or weeks. Although acetic acid is applied at relatively high concentrations, it does not have a long term negative influence on soil microorganisms. Acetic acid can also be used to control invasive aquatic weeds.

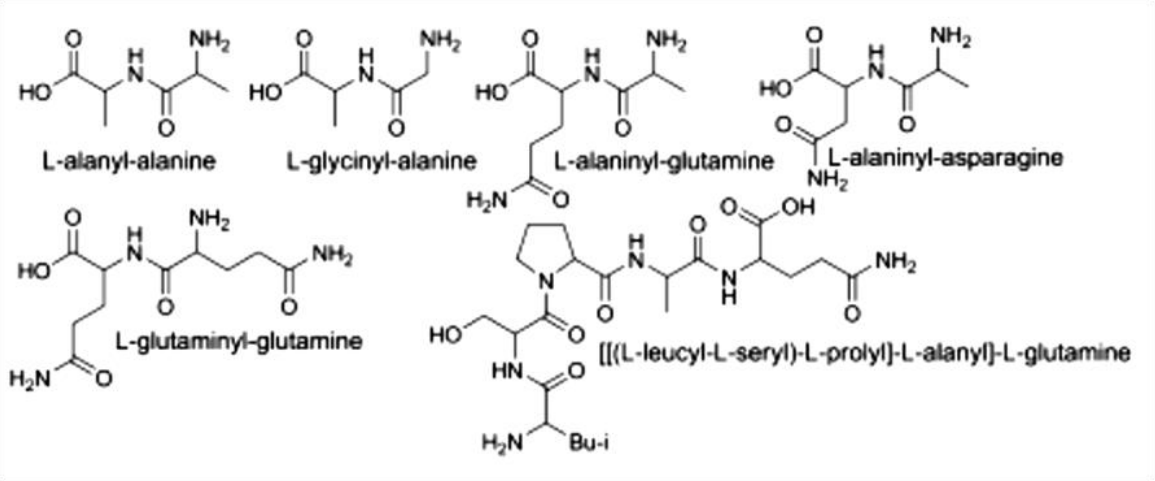


Figure 1.6 Structures of the phytotoxic oligopeptides in the hydrolisate of corn gluten meal used for weed management in organic agriculture (Dayan *et al*., 2009).

Fatty acids

The herbicidal activity of fatty acids has been known for many years (Malkomes, 2006). and some fatty acid salts are now marketed as non-selective herbicidal soaps. These are composed of fatty acids of various aliphatic length mixed with vinegar or acetic acid and emulsifiers such as organosilicone, saponified, methylated, and ethylated seed oil activator adjuvants. Herbicidal soaps act relatively rapidly and have no selectivity (broad-spectrum weed control). However, most weeds tend to recover because there is no residual activity after the initial burndown effect which takes place soon after application (Anonymous, 2002). As such, these mixtures can be used as desiccants. Fatty acids with midrange aliphatic tails such as caprylic (C8, octanoic acid) and pelargonic acid (C9) are the most effective (Coleman, 2006). Pelargonic acid is a contact, broadspectrum commercial herbicide. It disrupts plant cell membranes, causing rapid loss of cellular function. When saturated fatty acids from C6 to C14 were compared, the C9–C11 fatty acids were especially active, whereas the others were significantly less active (Fukuda *et al*., 2004). C6 and C14 fatty acids had essentially no herbicidal activity. Pelargonic acid itself is considered a low toxicity and environmental impact herbicide (Senseman, 2007; Malkomes, 2006). It has no residual activity. Adding organic acids such as succinic, lactic or glycolic acid enhance the efficacy of pelargonic acid formulations (Coleman *et al*., 2008).

Bialaphos

Bialaphos (Figure 1.7), a tripeptide obtained from the fermentation culture of the actinomycete *Streptomyces hygroscopicus*, is the only true commercialized natural product herbicide. It is a proherbicide that is metabolized into the active ingredient L-phosphinothricin (Figure 1.7) in the treated plant (Lydon *et al*., 1999). Bialaphos and phosphinothricin inhibit glutamine synthetase. Inhibition of glutamine synthetase, which is necessary for the production of glutamine and for ammonia detoxification, is lethal to plants. Plants exposed to glufosinate have reduced glutamine and increased ammonia levels in their tissues, which stops photosynthesis and results in plant death (Senseman, 2007; Duke, 2005). Phosphinothricin is translocated symplastically and apoplastically throughout treated plants and it is not susceptible to metabolic degradation.

While these are the only products available to have this mode of action, other natural products such as tabtoxine-b-lactam, oxetin, phosalacin and methionine sulfoximine (Figure 1.7) also target this enzyme (Lydon *et al*., 1999). Both bialaphos and phosphinothricin are broad-spectrum postemergence herbicides that can be used for total vegetation control in many agricultural settings, or in non-cultivated areas and to dessiccate crops before harvest.

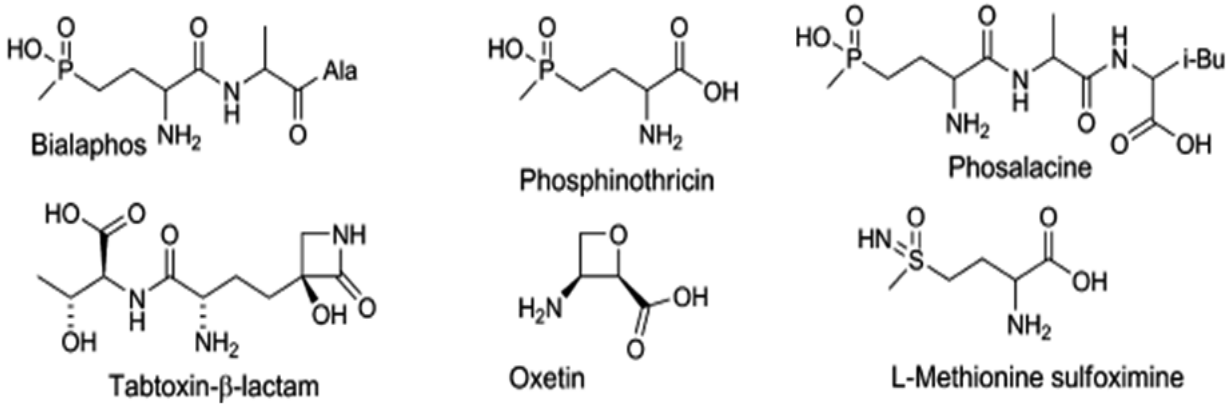


Figure 1.7 Structures of natural herbicides used in conventional agriculture and related compounds (Dayan *et al*., 2009).

1.2 TRP Active Natural Compounds

Nowadays naturally occurring substances, especially those derived from higher plants constitute an interesting resource in ecofriendly management of plant pests because they are 'generally recognized as safe' (GRAS). Plants have evolved ingenious defensive strategies to ward off herbivorous predators, worms and pathogenes. In many cases this is achieved through the production of secondary metabolites that determine irritation and inflammation (i.e. capsaicin, allicin, piperine, menthol). Cinnamon, clove, oregano and thyme essential oils reveled antibacterial activity towards several Gram-negative and Gram-positive pathogenic bacteria (Burt 2004; Azuma *et al*., 2003; Inouye 2003; Tajkarimi *et al*., 2010) and inhibited the growth of different phytopathogenic fungi (Soliman *et al*., 2002; Ranasinghe *et al*., 2002; Pawar *et al*., 2006; Feng *et al*., 2007; Christian *et al*., 2008; Kordali *et al*., 2008; Karbin *et al*., 2009; Siripornvisal *et al*., 2009). Extracts from garlic and mustard demonstrated also a wide range of antimicrobial property (Nielsen *et al*., 2000; Tedeschi *et al*., 2007; Khusniati *et al*., 2008; Sitara *et al*., 2008; Dimić *et al*., 2009; Shrestha *et al*., 2009; Škrinjar *et al*., 2009) and antielmintic activity (Gupta *et al*., 1993; Gupta *et al*., 2005; Flemming *et al*., 2006; Lazzeri *et al*., 2004). Pungent and irritant compounds produce their psychophysical effects by targeting excitatory transient receptor potential (TRP) channels. This fact is responsible of the chemosensation properties of the mentioned food plants, such as hotness, cooling, tingling, stinging, etc.

TRP channels are attracting much attention from various research areas including physiology, pharmacology and toxicology because of their variety of biological functions as well as their existence from yeast to mammals. From IPM point of view, TRP channel could be a possible new target site of pest control agents like natural toxin allylisothiocyanate, known also as insecticide (Nagata, 2007).

1.2.1 TRP family overview

TRP channels constitute a large and functionally versatile family of cation-conducting channel proteins, which have been mainly considered as polymodal unique cell sensors. The origin of the discovery of the TRP channels can be traced back to the 1960s, when a

Drosophila melanogaster mutant was found to show a transient response to prolonged bright light (Huang *et al.*, 2006).

The evolutionary first TRP channels in protists, chlorophyte algae, choanoflagellates, yeast, and fungi are primary chemo-, thermo-, or mechanosensors.

Many of these functions are remarkably conserved from protists, worms, and flies to humans. For instance, yeast use a TRP channel to perceive and respond to hypertonicity, nematodes use TRP channels at the tips of neuronal dendrites in their 'noses' to detect and avoid noxious chemicals. Male mice use a pheromone-sensing TRP channel to tell males from females. Humans use TRP channels in the perception of chemestetic sensations and to discriminate warmth and cold. In each of these cases, TRPs mediate sensory transduction, not only in a classical sense, for the entire multicellular organism, but also at the level of single cells (Clapham, 2003).

So far, some 70 TRP channels have been identified in both invertebrates and vertebrates. In sea squirts, nematodes and fruit flies, 30, 24 or 16 different TRP channels have been identified, respectively. In mammals, 33 different TRP channels have thus far been identified (Figure 1.8).

Based on amino acid sequence homology, the mammalian members of this family have been classified into 7 subfamilies. The TRPC (canonical) and TRPM (melastatin) subfamilies consist of seven and eight different channels, respectively (i.e., TRPC1–7 and TRPM1–8). The TRPV (vanilloid) subfamily presently comprises six members (TRPV1–6). The most recently identified subfamily, TRPA (ankyrin), has only one mammalian member (TRPA1). The TRPP (polycystin) and TRPML (mucolipin) families, each containing three mammalian members, are relatively poorly characterized, but are attracting increasing interest because of their involvement in several human diseases. The TRPN subfamily (NOMP, No mechanopotential) in hearing-assisting sensory neurons in *Drosophila* and zebrafish (*Danio rerio*) has to date only been detected in worm, *Drosophila,* and zebrafish and is proposed to be a mechanostimuli sensing channel. Currently available genome information indicates that mammals have no TRPN orthologs (Nilius *et al*., 2007).

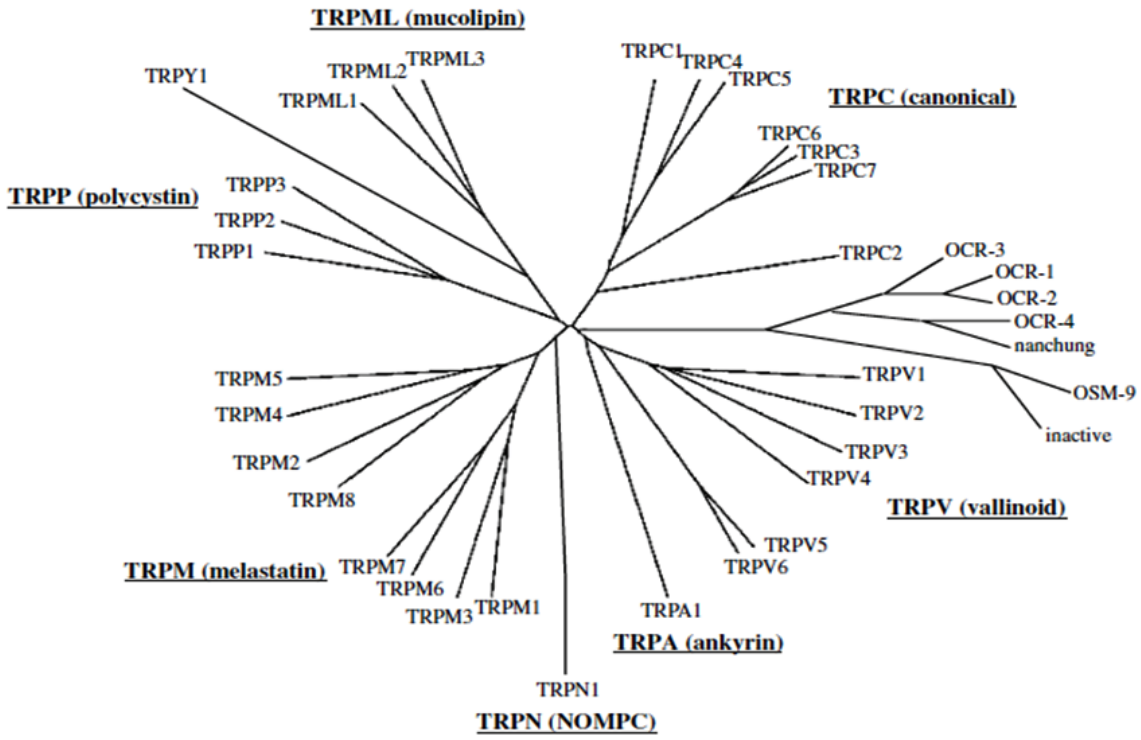


Figure 1.8 Unrooted phylogenetic tree generated by aligning the transmembrane domains of the TRP channels. The seven main branches are denoted with underline, the letters and numbers following TRP indicate TRP subfamily and member, respectively (Yin *et al*., 2010).

1.2.2 Mammalian TRP channel structure

Typically, each TRP channel subunit consists of six putative transmembrane spanning segments (S1–6), implanted with a pore-forming loop between S5 and S6, and intracellularly located cytoplasmic NH_2 and COOH termini. Assembly of channel subunits as homo- or heterotetramers results in the formation of cation-selective channels (Figure 1.9). Cations are selected for permeation by the extracellular-facing pore loop, held in place by the S5 and S6 a-helices. All TRP channels are nonselective, with the exception of the monovalent-selective TRPM4 and TRPM5, and the Ca^{2+}-selective TRPV5 and TRPV6. The cytoplasmic ends of the S6 helices form the lower gate, which opens and closes to regulate cation entry into the channel. The selectivity filter itself may also gate. The S1–S4 domain may flex relative to S5–S6 in response to stimuli, but the paucity of positively charged arginines in TRP S4 helices indicates weak voltage sensitivity. All elements outside the S5–S6 region provide means of either subunit association or act as linkers to elements that control gating (Clapham, 2003) .

The selectivity filter (light blue and inset) is formed by amino acids that dip into the bilayer (pore loops), one contributed from each of the four subunits. Depending on the TRP family, the N-terminus contains between zero and eight ankyrin repeats, a predicted coiled coil region, and a putative caveolin-binding domain. The C-terminus comprises a TRP signature motif (EWKFAR), a proline-rich motif, the calmodulin/inositol 1,4,5-trisphosphate (IP3) receptor-binding (CIRB) domain, and a predicted coiled coil region (Yin *et al*., 2010), (Figure 1.10).

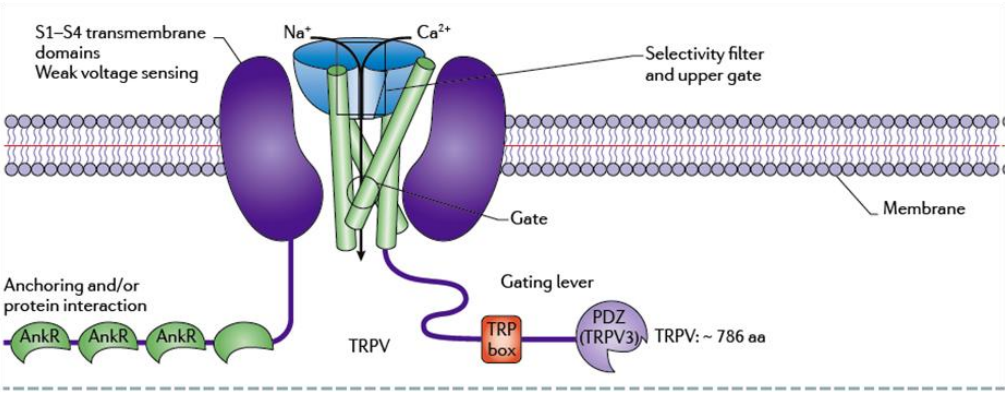


Figure 1.9 TRP channel architecture (Moran *et al*., 2011).

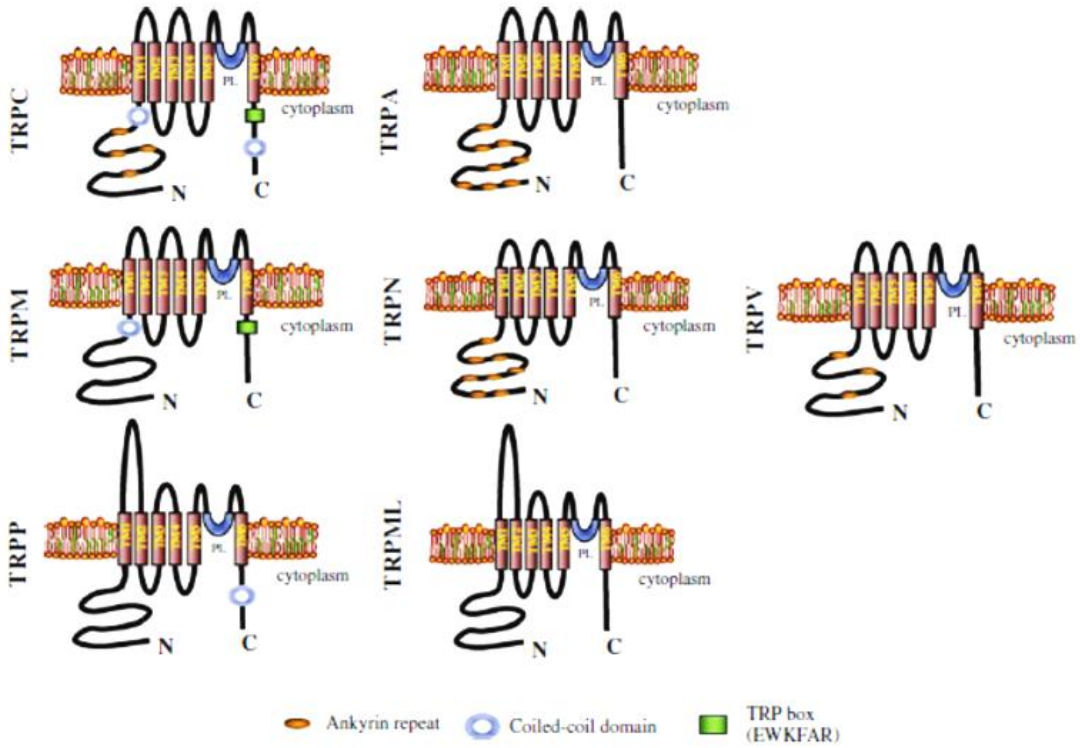


Figure 1.10 Simplified schematic structure sketches of the seven subfamilies of TRP channels. The following domains are indicated as TM transmembrane segments, PL pore loop. Other domains as ankyrin repeats, coiled-coil domain, and TRP box are depicted as shown (Yin *et al*., 2010).

The activation and regulation mechanisms of TRP channels are largely unknown and highly diverse. Channel activity is affected by several physical parameters such as osmolarity, pH, mechanical force, as well as biochemical interactions with external ligands or cellular proteins. Nonetheless, the established modes of activation for expressed TRP channels may be divided into three general categories:

1. *Receptor activation.* G protein–coupled receptors (GPCRs) and receptor tyrosine kinases that activate phospholipases C (PLCs) can modulate TRP channel activity in at least three ways: (*a*) hydrolysis of phosphatidylinositol bisphosphate (PIP2), (*b*) production of diacylglycerol (DAG), or (*c*) production of inositol trisphosphate (IP3) and subsequent liberation of Ca^{2+} from intracellular stores.

2. *Ligand activation.* Ligands that activate TRP channels may be broadly classified as (*a*) exogenous small organic molecules, including synthetic compounds and natural products (capsaicin, icilin,); (*b*) endogenous lipids or products of lipid metabolism (diacylglycerols, phosphoinositides, eicosanoids, anandamide); (*c*) purine nucleotides and their metabolites [adenosine diphosphoribose (ADP-ribose), *β*NAD+]; or (*d*) inorganic ions, with Ca^{2+} and Mg^{2+} being the most likely to have physiological relevance. Although some TRP channels clearly function as chemosensors for exogenous ligands (i.e., capsaicin activation of TRPV1), relatively few endogenous chemical ligands with the capacity to activate TRP channels from the aqueous extracellular milieu are known (2-AG, anandamide).

3.*Direct activation.* Changes in ambient temperature are strongly coupled to the opening of TRPV1–TRPV3 and TRPM8 by poorly understood mechanisms. Other putative direct activators include mechanical stimuli, conformational coupling to IP3 receptors, and channel phosphorylation. Heating and cell swelling may also act indirectly to activate TRP channels through second messengers or other unidentified mechanisms (Ramsey *et al*., 2006).

1.2.3 TRP receptors and chemosensation

A large variety of plant-derived natural products and other chemical agents evoke sensory responses with an infinite shade of perceptual qualities. The perception of chemical stimuli by sensory means is referred to as chemosensation or chemoreception. In humans, the olfactory and the gustatory systems are the principal chemosensory systems and the substrates for the senses of smell and taste, respectively. Although less well recognized, the trigeminal somatosensory system also plays a fundamental role in chemosensation.

Sensory endings of the trigeminal (V cranial) nerve innervate the skin covering the face, the mucous membranes of the nasal and oral cavities, and the cornea and conjunctiva of the eye. These endings can be activated by physical stimuli (mechanical forces and temperature) and by a huge array of chemical agents, and evoke sensations of touch, temperature, and pain. The capacity of cutaneous, including trigeminal endings, to detect chemicals is known as chemesthesis or cutaneous chemosensation (Viana, 2010). Oral chemesthesis explains the pungent or sharp feel of many different foods and spices such as chili peppers, horseradish, wasabi roots, and Szechuan pepper, the coolness of peppermint, the tingle of carbonated drinks, and the irritation produced by substances such as nicotine or raw garlic extracts.

In the trigeminal system, the sensations evoked by many chemical agents show clear cross interactions with temperature. Many chemical agents (e.g., capsaicin) sensitize the perception of temperature. Menthol sensitizes responses of trigeminal endings to cold temperature. These interactions are easily explained by the allosteric gating of TRP channels by chemical and thermal stimuli. TRP channels with a marked sensitivity to temperature are referred to as thermoTRPs (Viana, 2010), (Figure 1.11).

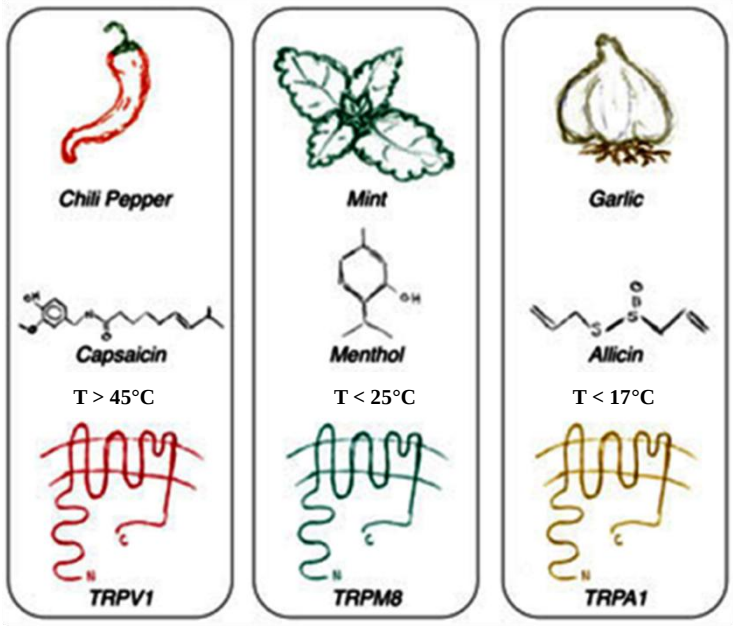


Figure 1.11 Schematic of natural plant products and their related receptors (Gerhold *et al*., 2009).

1.2.4 TRPV1

TRPV1, originally named vanilloid receptor 1 (VR1) and commonly referred as the capsaicin receptor, was first described as a polymodal receptor activated by three pain-producing stimuli; vanilloid compounds (capsaicin, resiniferatoxin), moderate heat (≥43 °C) and low pH (<5.9). Since then, TRPV1 has been reported to be also activated by camphor, allicin, nitric oxide, spider toxins, potentiated by ethanol and modulated by extracellular cations. TRPV1 was initially described in a subpopulation of small-to medium-diameter neurons in dorsal root, trigeminal and nodose ganglia. While TRPV1 has since been described in many other neuronal and non neuronal cells, its highest expression level is in sensory neurons (Levine *et al*., 2007). An important functional characteristic of TRPV1 channels is their high permeability to Ca^{2+} ions. In addition, prolonged exposure of TRPV1 to vanilloid agonists results in a modification of the ionic pore properties (i.e., "pore dilation") with a threefold increase in the relative permeability to Ca^{2+} (as compared to sodium). This results in a Ca^{2+} overload of capsaicin-sensitive nerve terminals, the retraction of epidermal nerve fibers, and the long-lasting functional desensitization of nociceptive sensory endings.

1.2.4.1 Natural products ligands of TRPV1

Capsaicinoids

The fruits of *Capsicum* plants are commonly known as chili peppers and their use in culinary preparations and in traditional medicine is known since many centuries. TRPV1 is activated by capsaicin (compound **1** in Figure 1.12), the pungent component of "hot" chilli peppers. Capsiate is a nonpungent capsaicin analog, obtained from a non-pungent cultivar of red peppers (as *Capsicum annuum*). Several studies have reported on the pungency and bioactivity of various capsaicinoids. The non-pungency of capsiate has been related with its poor accessibility to sensory neurons when administered to skin or mucosa by its degradation and trapping in the lipid phase of epithelium or cornea due to its high lipophilicity.

Piperine

Piperine is an alkaloid found naturally in plants belonging to the *Piperaceae* family, such as *Piper nigrum*, commonly known as black pepper, and *Piper longum*, also known as long pepper. Black pepper has been used in traditional Chinese medicine to treat seizure disorders. It has putative anti-inflammatory activity and may have activity in promoting digestive processes. Piperine (compound **2** in Figure 1.12) binds to and activates TRPV1. Although approximatively 200fold less potent than capsaicin for the activation of hTRPV1, piperine shows nevertheless a remarkable efficacy, higher than that of capsaicin. Furthermore, and just like camphor, piperine shows very potent desensitizing activity, suggesting that, for TRPV1, potency and desensitization can be dissociated (Appendino *et al.*, 2008).

Eugenol

Eugenol (compound **3** in Figure 1.12) is an allyl chain-substituted guaiacol, i.e. 2-methoxy-4-(2-propenyl)phenol and is a member of the allylbenzene class of chemical compounds. Eugenol is the chief constituent of clove or clocimum oil obtained from *Eugenia carophyllata* and *Ocimum gratissimum*. After isolation of eugenol from clove oil, it was demonstrated in electrophysiological studies, that eugenol is able to activate inward currents in hTRPV1-HEK293 cells and TG neurons. However, other TRP channels could also be activated by eugenol.

Resiniferanoids

The extremely irritant diterpene present in the dried latex of the plant *Euphorbia resinifera*, resiniferatoxin (RTX, compound **4** in Figure 1.12) is a very specific agonist for the TRPV1 channel. *E. resinifera* is a cactus-like plant native to the Anti-Atlas Mountains of Morocco. Early reports of the medical use of dried latex of *E. resinifera* describe its direct application to dental cavities to mitigate toothache or to suppress chronic pain. Because capsaicin and RTX analogues share a homovanillyl group as a structural feature motif essential for bioactivity, these naturally occurring substances were collectively called vanilloids.

Importantly, RTX is about 3 to 4 magnitude more potent than capsaicin as well in dose-response curve as for the effect on thermoregulation and neurogenic inflammation.

Ginger-Derived Products

These classes of compounds were isolated from ginger plants (*Zingiber officinale*), a reed-like plant. Ginger though called a root, is actually the rhizome of the monocotyledonous perennial plant *Zingiber officinale*. Ginger contains up to 3% of an essential oil that causes the fragrance of the spice. The main constituents are gingerols, such as [8]- and [6]-gingerol, other pungent substances, are shogaol, zingerone, and paradol. Gingerols ([6,8,10]-gingerols) and shogaols ([6,8,10]-shogaols) have differences in the length of the alkyl carbon chain. Gingerols and shogaols are both activators of the TRPV1 channel. Zingerone is similar in chemical structure to other flavor chemicals such as vanillin and eugenol. Fresh ginger does not contain zingerone; cooking the ginger transforms gingerol, which is present, into zingerone (compound **5** and **6** in Figure 1.12, respectively). Out of experimental data, it has been suggested that capsaicin and zingerone could activate the same receptor. Cultured rat TG neurons and TRPV1-*Xenopus* oocytes were desensitized by repeated applications of zingerone. Moreover, analysis in rTRPV1-HEK 293 cells showed that gingerols increased intracellular Ca^{2+}. In this way, zingerone and gingerols represents a class of naturally occurring TRPV1 receptor. Paradol can be obtained from gingerol by successive dehydration and hydrogenation and is also found in the seeds of *Aframomum melegueta* as a major pungent principle. [6]-Paradol has been cited as pungent, but little is known about its possible direct activation of TRPV1.

Camphor

Despite a long history of medicinal use as a topical analgesic, the mechanism of activity of the monoterpenoid camphor (compound **7** in Figure 1.12) has remained unknown until recently. Camphor activates TRPV3, but repeated applications *sensitize* rather than *desensitize* TRPV3, showing that other mechanisms must be involved in its analgesic properties. Although camphor was much less potent than capsaicin for the activation of TRPV1, it could nevertheless desensitize TRPV1 more rapidly and completely than capsaicin, an observation that provides a rationale for the use of this monoterpenoid in plasters and other topical preparation against pain and inflammation.
Camphor could also inhibit TRPA1, an activity that synergizes with the desensitization of TRPV1 to induce analgesia.

Cannabinoids

Cannabinoids are a group of chemicals that referred to an unique group of secondary metabolites found in the cannabis plant, *Cannabis sativa*, which are responsible for the peculiar pharmacological effects of the plant. *Cannabis* species contain a complex mixture of substances that include 60 different cannabinoids, of whom tetrahydrocannabinol (THC), cannabidiol (CBD) and cannabinol (CBN) are the most prevalent. Cannabinol (compound **8** in Figure 1.12) is the primary product of tetrahydrocannabinol degradation, and there is usually little of it in a fresh plant. CBN content increases as THC degrades in storage, and with exposure to light and air. It is only mildly psychoactive, and is perceived to be sedative or stupefying. The biological actions of cannabidiol cannot be exclusively related to its cannabinoid receptor interaction, considering its low affinity (micromolar range) for cannabinoid receptors. Interestingly, some actions of cannabidiol are similar to those of capsaicin, including anti-inflammatory and analgesic effect. Interaction with TRPV1 seems to be related to some *in vivo* effects of cannabidiol, since it was shown that the TRPV1 receptor mediates the analgesic action of cannabidiol.

Evodia Compounds

Evodiamine (compound **9** in Figure 1.12) is a chemical which is extracted from fruits of *Evodia rutaecarpa*. In traditional Chinese, and Japanese medicine *Evodia* fruits have been prescribed for the treatment of headache, thoraco-abdominal pain, and vomiting that are caused by cold temperatures. Its mode of action is believed to be similar to that of capsaicin, since evodiamine is indeed a genuine agonist of TRPV1. It produces extracellular Ca^{2+} uptake as well as intracellular Ca^{2+} increase in rat TRPV1 expressing cells, and both effects are competitively antagonized by capsazepine, a capsaicin antagonist and a blocker of TRPV1. Evodiamine shows the analgesic action by desensitizing sensory nerves. It should be mentioned however that evodiamine doesn't taste hot, indicating that it's above mentioned effects might be mediated through its interaction with another factor other than the TRPV1 channel.

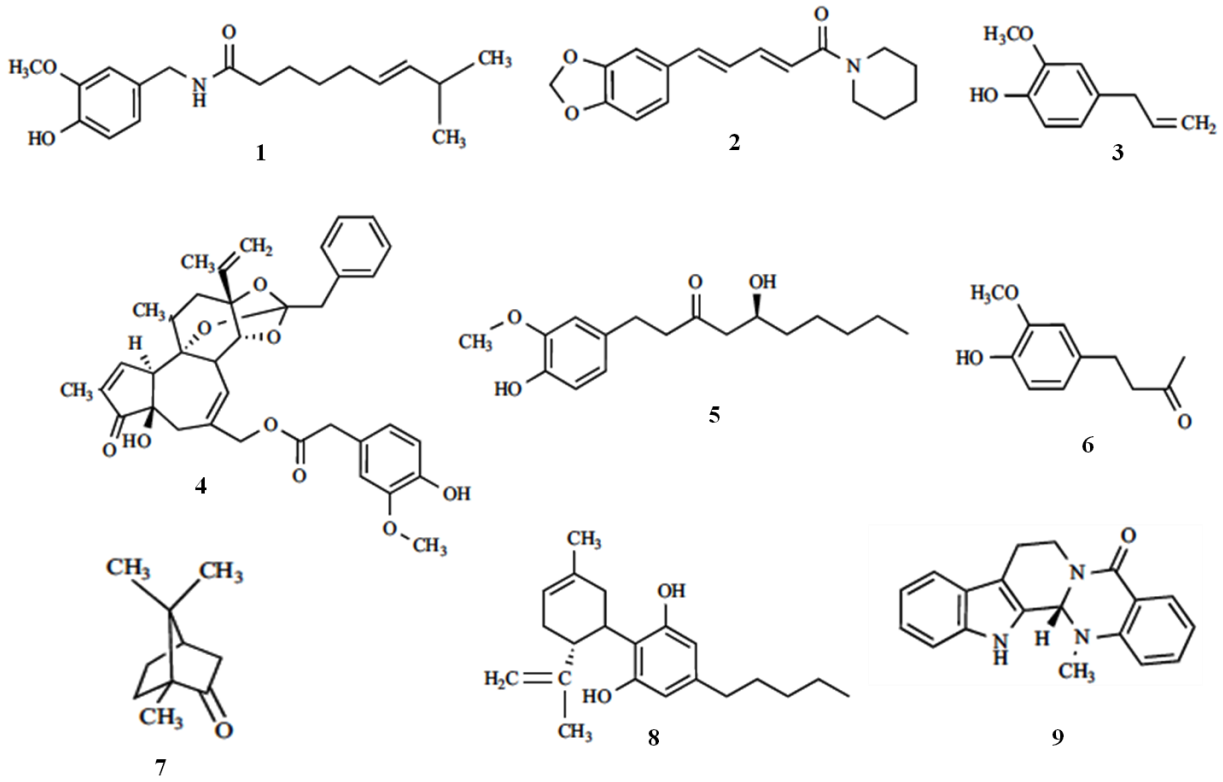


Figure 1.12 Structures of some TRPV1 agonists (Vriens *et al*., 2008).

1.2.5 TRPV2

TRPV2 is an ion channel with an amino acid sequence ~50% identical to TRPV1. It is expressed in sensory neurons of medium and large diameter. The channel is insensitive to capsaicin and low external pH. TRPV2 is activated by noxious heat, with an activation threshold (~52°C) higher than that of TRPV1 and to changes in osmolarity. Very few natural chemical agonists of TRPV2 are known, and none are considered selective. Δ9-tetrahydrocannabinol and cannabidiol of the marijuana plant activate the channel.

1.2.6 TRPV3

TRPV3 is an ion channel structurally analogous to TRPV1 that is strongly expressed in skin keratinocytes. Expression of TRPV3 in peripheral sensory neurons is a controversial subject. When transfected into mammalian cells, this channel responds to temperature with a threshold around 33°C but not to capsaicin or pH changes. TRPV3 null mice have strong deficits in responses to innocuous and noxious heat, indicating that it participates in thermosensation.TRPV3 is activated by a number of natural chemical compounds,

including camphor and carvacrol, a monoterpenoid phenol present at high concentrations in the essential oil of oregano. Other monoterpenes such as eugenol (derived from cloves), dihydrocarveol, thymol (from thyme), carveol, and (+)-borneol also activate TRPV3. Incensole acetate, a compound in the resin obtained from *Boswellia* trees, also activates TRPV3. Farnesyl pyrophosphate (FPP), an intermediate metabolite in the mevalonate pathway, is a specific activator of TRPV3. The cutaneous sensations produced by heating and cooling can be enhanced by camphor coapplication.

1.2.7 TRPM8

The transient receptor potential melastatin 8 channel (TRPM8) is a nonselective cation channel activated by mild cold temperatures (threshold around 25°C in heterologous systems) and cooling compounds such as menthol. The TRPM8 protein is 1104 amino acids long and is found in a subpopulation (10-15%) of small-diameter, cold-sensitive peripheral sensory neurons. The same neurons are activated by cooling compounds (Figure 1.11). Thermosensitive nerve endings of these sensory neurons innervate the skin and mucosae (cornea, oral cavity) where TRPM8 plays a clear physiological role in the detection of low temperature ambient signal. In addition to cold, TRPM8 can be activated by natural and synthetic cooling mimetic agents such as icilin, eucalyptol, menthol, and an abundance of menthol analogues. Other natural weak agonists of TRM8 include hydroxy-citronellal, geraniol, and linalool.

1.2.8 TRPA1

TRPA1 (before ANKTM1) is a nonselective cationic TRP channel (1119 amino acids in humans), phylogenetically distant to other mammalian TRP proteins. Its C-terminus contains as many as 14 ankyrin binding domains. TRPA1 is expressed in small-diameter neurons of the trigeminal and dorsal root ganglia, but apart from that also in hair cell epithelium. TRPA1 is activated by cold temperatures (<17°C) and by an intriguing set of natural products (Table 1.2), including pungent chemicals as isothiocyanates occurring naturally in fruits and plants such as mustard greens and capers. Most notably, the chemical sensitivity of this receptor has been preserved during evolution, being similar in humans and flies. The list of TRPA1 agonists keeps growing daily and includes many natural and synthetic irritants such as allyl isothiocyanate, cinnamaldehyde, allicin and

diallyl disulfide, methyl salicylate, ginger, carvacrol, formalin, natural fungal deterrents like isovelleral, unsaturated aldehydes like acrolein, isocyanates, and oxidizing agents like hypochlorite (OCl^-) and hydrogen peroxide (H_2O_2). Several odorants (R-terpineol, amyl acetate, benzaldehyde, toluene) known to activate trigeminal fibers also activate TRPA1 channels. The unique chemical sensitivity of this ion channel for some of these irritants was clarified in TRPA1 knockout mice. These animals were completely insensitive to mustard oil and allicin.

1.2.8.1 Natural products ligands of TRPA1

Isothiocyanate compounds and Cinnamaldehyde

Isothiocyante derivatives constitute the main pungent ingredients in wasabi (allyl isothiocyanate), yellow mustard (benzyl isothiocyanate), Brussels sprouts (phenylethyl isothiocyanate), nasturtium seeds (isopropyl isothiocyanate) and capers (methyl isothiocyanate). Allyl isothiocyanate is the major active ingredient in mustard oil. Topical application of mustard oil to the skin activates underlying sensory nerve endings, thereby producing burning pain, inflammation and robust hypersensitivity to both thermal and mechanical stimuli, but the mechanism through which these compounds elicit their effects was unknown until recently. Calcium imaging and electrophysiological analysis showed that each of the above mentioned compounds was capable of activating human TRPA1, expressed in oocytes. Concomitantly, allyl thioisocyanate elicits a Ca^{2+} response in subset of cultured neurons trigeminal and dorsal root ganglia, which is dependent on extracellular Ca^{2+}, indicating that a Ca^{2+} influx channel is involved.

Importantly, this response is completely lacking in Trpa1 knockout mice, indicating that TRPA1 is the sole target through which mustard oil activates primary afferent nociceptors.

Cinnamaldehyde is the main constituent isolated from cinnamon oil which is the essential oil obtained from *Cinnamomum cassia* or *Cinnamomum zeylanicum*.

It is routinely used for flavouring purposes, and in human subjects, cinnamaldehyde is reported to elicit a burning and tingling sensation in the mouth. Notably, no response was obvious when cinnamaldehyde was applied to TRPV1, TRPM8 and TRPV4 expressing cells. When injected, cinnamaldehyde elicits pain-related behavior in mice. As expected this response is similar in Trpv1 knockout mice. Further testing in Trpa1 knockout mice has not yet been reported.

Other Natural Products

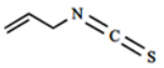
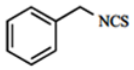
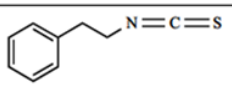
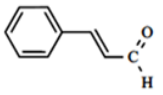
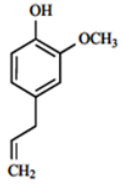
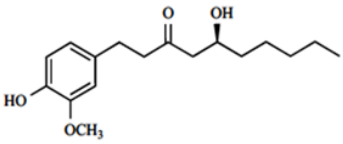
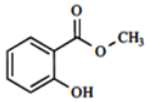
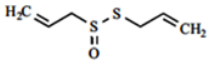
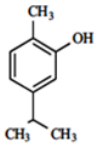
TRPA1 overexpressing cells show a sharp increase in intracellular free Ca^{2+} levels when stimulated with plant derived compounds such as eugenol (from clove oil), gingerol (from ginger) and methyl salicilate (from wintergreen oil). All these compounds cause a pungent burning sensation in humans. Allicin, an unstable compound of fresh garlic, is the chemical responsible for TRPA1 and TRPV1 activation. It is commonly known that raw garlic elicits burning pain and prickling sensations on the lips and the tongue. Trigeminal and dorsal root ganglion neurons from TRPA1 knockout mice were completely insensitive to allicin, indicating that TRPA1 is the sole site of action of these compounds. Another plant derived compound activating TRPA1 is carvacrol, the major ingredient of oregano. TRPA1 is rapidly activated and desensitized by this compound, as is however also TRPV3.

1.2.8.2 Mechanisms of TRPA1 activation

The mechanism of ligand activation of TRPA1 may involve either covalent modification of cysteine residues or conventional reversible ligand-receptor interactions (Hongzhen, 2010).

In the covalent binding the reactive carbons of ligands form Michael adducts by binding with specific N-terminal cysteine residues on TRPA1, in particular the α, β-unsaturated carbonyl groups of the ligands react with the -SH groups of cysteines on the channel. TRPA1 is activated by structurally different molecules with high chemical reactivity (Bang *et al*., 2009). These covalent modifications produce long lasting channel openings that can be reversed by reducing agents such as dithiothreitol. Mutational studies have identified several cysteine and lysine residues involved in channel activation. In humanTRPA1, crucial residues for channel activation by allyl isothiocyanate include a cluster of cysteines (Cys619, Cys639, and Cys663) and Lys708. Actually, activation of TRPA1 by electrophilic compounds is very complex. For example, certain Cys-reacting compounds show species-specific responses in TRPA1 channels. Other agonists of TRPA1 appear to act by conventional reversible ligand-receptor interactions such as eugenol and carvacrol. Mutating the critical cysteines to nonreactive serines does not prevent the agonist action of these compounds (Viana, 2010).

Table 1.2 Natural products ligands of TRPA1 (Vriens *et al*., 2008).

Compound	Structure	Origin	Action	Conc. Range
Allyl-isothiocyanate		Wasabi, mustard oil	Activates	$10^{-5} - 10^{-6}$M
Benzyl- isothiocyanate		Yellow mustard	Activates	$10^{-4} - 10^{-5}$M
Phenylethyl- isothiocyanate		Brussels sprouts	Activates	$10^{-4} - 10^{-5}$M
Isopropyl- isothiocyanate		Nasturtium seeds	Activates	$10^{-4} - 10^{-5}$M
Methyl- isothiocyanate	CH_3NCS	*Capparis spinosa*	Activates	$10^{-4} - 10^{-5}$M
Cinnamaldehyde		*Cinnamomum cassia* or *Cinnamomum zeylanicum*	activates	$10^{-5} - 10^{-6}$M
Eugenol		Clove oil	Activates but also TRPM8 and TRPV1	$10^{-3} - 10^{-4}$M
Gingerol		*Zingiber officinale*	Activates	$10^{-3} - 10^{-4}$M
Methyl salicilate		Wintergreen oil	Activates	$10^{-3} - 10^{-4}$M
Allicin		*Allium Sativum*	Activates but also TRPV1	$10^{-3} - 10^{-4}$M
Carvacrol		*Origanum* genus	Activates	$10^{-3} - 10^{-4}$M

1.3 *Perilla frutescens*

Perilla [*Perilla frutescens* (L.) Britton] is a short-day annual herb belongs to family Labiateae (Lamiaceae). It is a traditional crop of some Asian countries such as China, Korea and Japan. Although the wild ancestor of *Perilla* crop has not yet been identified, the original birth place of *Perilla* crop is considered to be mountainous areas of China, because of long history of cultivation.“Ch'i-min-yao-shu”, written in the fifth or sixth century in China, is the oldest literature in which the perilla crop is described. Seed remains of perilla have been also found from several archaeological sites in Japan dated ca. 5000 B.P. Perilla seeds were found in carbonized starch cakes found in Nagano, Gifu and Fukushima prefectures of Japan. These seeds were probably used for flavoring foods, as were nuts and sesame seeds in the past. (Nitta *et al*., 2003). Since ancient times, perilla has been known as an herbal medicine, vegetable, garnish, flavoring, and natural colorant in Asian countries. It was gradually recognized worldwide as a new economic plant with multiple uses. Recently the species has also been cultivated in many European and North American countries for medicinal and culinary uses (Lee *et al*., 2006). Perilla easily adapt to open sunny fields with humid climate. The prefer environment for cultivation is a well-drained rich soil with light to medium moist and full sun. Generally the cold hardened seeds are sown in pots filled with sifted compost consisting of loam, leaf mold and sand and covered lightly with soil. In warm and humid weather, plants grow quickly and the mature plants may grow about one meter high and are bushy and self-branching. Weedy plants grow on roadsides and abandoned fields.

1.3.1 Botanical characterization

Perilla frutescens has opposite leaves and branches, square stems, and flowers are irregular in shape and purple or white in color. The indeterminate inflorescences produce from all active meristems along the stem and branches with terminal raceme 6-20 cm long. The flower is small with pubescent campanulate calyx and lipped corolla. The corolla has five lobes, two upper and three lower (Figure 1.13). With the simple vascular architecture, leaves and branches emerge on the same side of the square stem share vascular bundles, leaves and branches on adjacent sides share half of their bundles, while leaves and branches on the opposite sides have no bundles in common. The leaves are fuzzy, dark

purple or green and flowers are self-pollinated without insect visits. To maintain a neat appearance, their tops are usually pinched off when the fourteenth primary leaf appears on the main stem. As a short-day summer annual, the flowering of perilla is sensitive to change in day length. It blooms in October and is killed by frost in winter locally (Lee *et al*., 2009). Fruiting time is July to October and the ripe fruit is a collection of grayish-brown nutlets containing 1-4 granules of seed. The seeds are ovoid or subspherical, about 0.6-2 mm in diameter, greyish-brown to blackish-brown, and with a net-patterned testa.

Figure 1.13 *Perilla frutescens*: a) habit, b) calyx, c: corolla, d) nutlet (Dönmez, 2002).

The genus *Perilla* contains two distinct varieties classified on the basis of their typical fragrance, morphologic characteristics and utilization. *P. frutescens* var. *frutescens* is used as an oil crop and as a common traditional vegetable particularly in Korea. It is known as Kkaennip in Korea, Egoma in Japanese, and Ren in Chinese (Figure 1.14). *P. frutescens* var. *crispa*, is utilized for medicinal or nutritional purposes above all in Japan.

The common names are Cha-jo-ki in Korean, Shiso in Japanese, and Zisu in Chinese (Jung et al., 2008). *P. frutescens* var. *frutescens* is taller (above 2 mm), larger in seed size, has either soft or hard seeds, is green in the leaves and stem, and has generally large, round, flat leaves, with a less serrate edge and often, a violet coloring on the reverse side.

P. frutescens var. *crispa* is smaller in plant height and seed size (below 2 mm), has only

hard seeds, is red or green in the leaves and stem, has wrinkly or non-wrinkly leaves (Lee *et al*., 2003). The green leaf type of perilla, called ao-shiso in Japan, is a popular herb in the Orient. The red (or purple) type of perilla, called aka-shiso in Japan, is wealthy in anthocyanin and a number of chemical compounds and often used as garnish, flavoring and colorant (Figure 1.14).

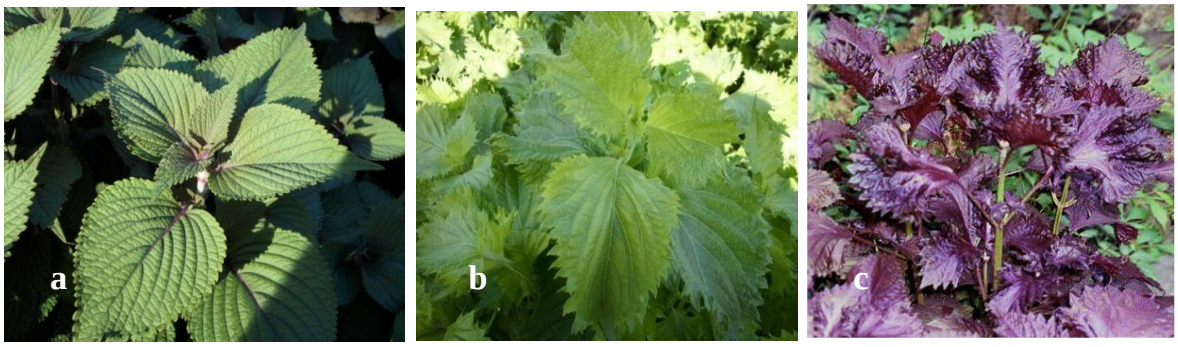


Figure 1.14 Different varieties of *Perilla frutescens*: a) Kkaennip, b) ao-Shiso, c) aka-Shiso.

1.3.2 Chemotypes: variations in chemical components

Perilla plants have a characteristic odor derived from their variety of essential oil components. Essential oil, consisting of monoterpenoids or phenyl propenes, is accumulated in the glandular trichomes on the surface of stems and leaves (Ito *et al*., 2008). The composition of perilla essential oil has been extensively investigated.

The essential oil of *Perilla frutescens* from different countries, Thailand, Turkey, northern Laos, Korean and Lithuania, were investigated. Species, chemotypes, geographical location, and growing environment are the main factors that affect the essential oil composition (Huang *et al*., 2011). According to the type and content of the principal volatile compounds, perilla can be classified into eight chemotypes: PK (perillaketone), DLP (D-limonene and piperitone), PT (piperitenone), MS (myristicin), AL (apiol), EM (elemicin), DEK (dehydroelsholtzia ketone) and PA (perillaldehyde) (Zhang *et al*. 2009). Figure 1.15 shows a theoretically scheme proposed by Ito *et al*. (2008) for the biosynthesis of components of *Perilla frutescens* essential oil. Perillaldehyde is the major component found in the essential oil of *P. frutescens* var. *crispa*. This cyclic monoterpenoid, soluble

in alcohol and insoluble in water, is an intermediate for synthesis of the intensive sweetener perillartine and it is used as food additives for flavourings and in perfumery to add spiciness (Huang *et al*., 2011). Perillaketone, a terpenoid component present in the leaves and seeds of Korean perilla, consists of a furan ring with a six-carbon side chain. It is a toxic compound found in the plant, which has been demonstrated to cause acute restrictive lung diseases in some animals as horses, cattle and sheep (Huang *et al*., 2011; Seo *et al*., 2009). Although perilla is ordinarily avoided by livestock, it is a popular leafy vegetable considered safe for humans, which is generally consumed as a pickle or wrapping with roasted.

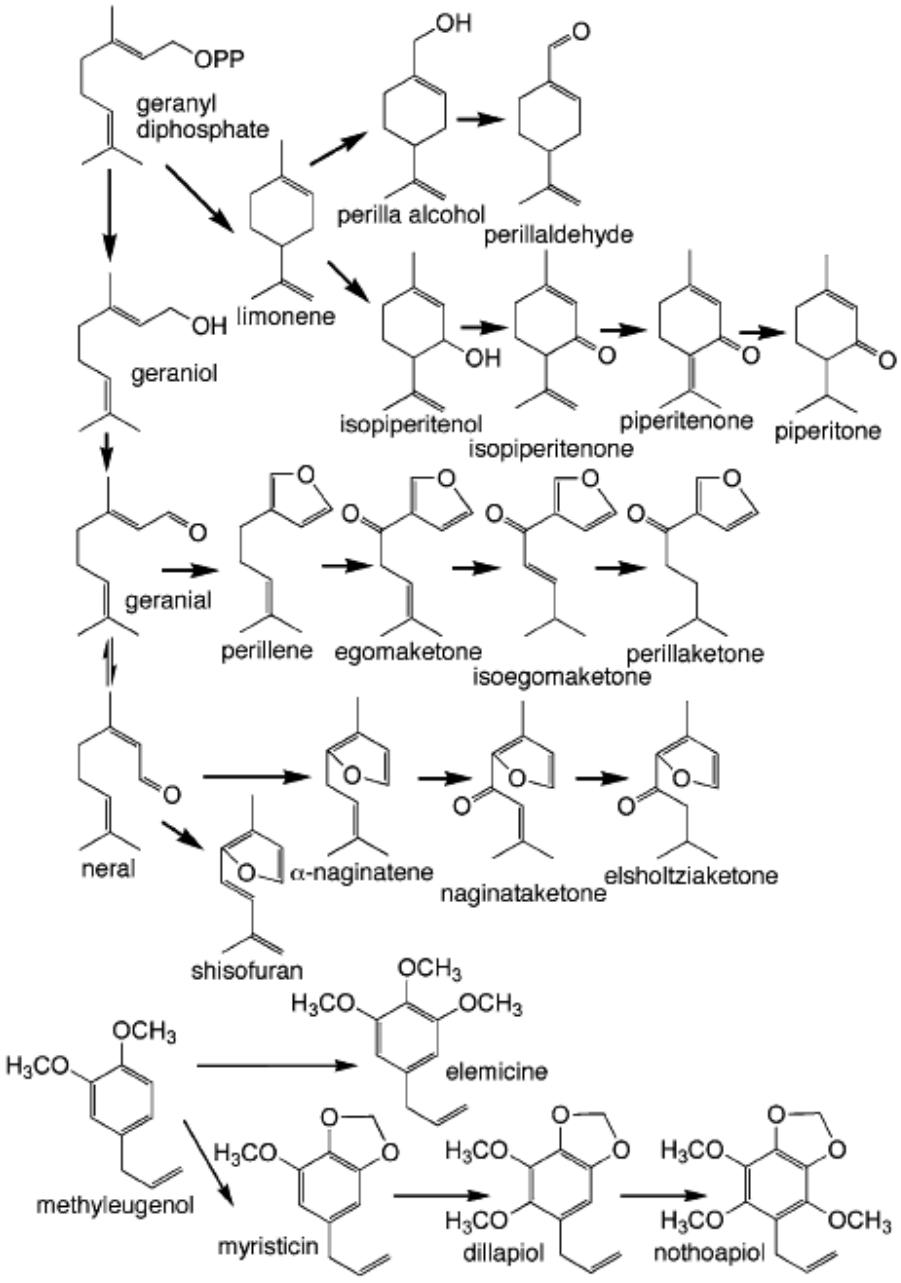
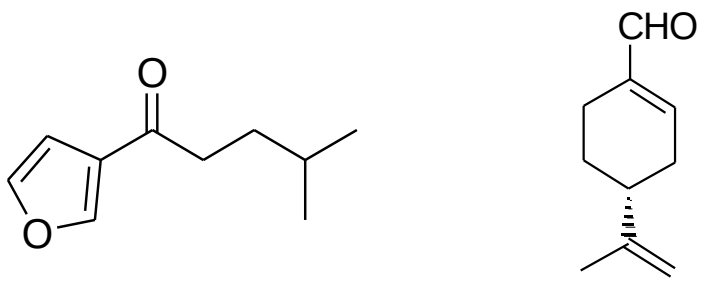


Figure 1.15 Proposed biosynthetic pathways for some essential oil components of *Perilla frutescens* (Ito *et al*., 2008).

1.3.3 Activation of TRPA1 receptor by PA and PK

The essential oil is mostly responsible for the aroma and taste of *Perilla frutescens*. Laureati *et al*., (2010) investigated the sensory properties of three different cultivars of perilla by means a panel of trained assessors and instrumental sensory devices such as electronic nose and electronic tongue. Results showed a remarkable difference of the three cultivars. The Korean variety of perilla was perceived as significantly less bitter and most refreshing (high intensity of cooling sensation). The crisp green-leaved perilla was the most aromatic sample being characterized by high intensity of grassy odour and flavour and by floral odour, while the red-leaved perilla was perceived as significantly less astringent and pungent as compared to the other two varieties of perilla. The fact that all the varieties were characterized by trigeminal sensations seems to confirm the outcome on in vitro assays obtained by Bassoli *et al*., (2009) who evidenced that two of its major secondary metabolites, PA and PK (Figure 1.16), isolated from fresh and freeze-dried perilla leaves are able to activate the cloned TRPA1 receptor.

Using a fluorometric test, they showed that rat TRPA1-HEK293 cells exhibit a sharp increase in intracellular $[Ca^{2+}]_i$ upon application of PK. The activity of the compounds was normalised to the maximum intracellular Ca^{2+} elevation generated by application of allylisothiocyanate (mustard oil, MO) 100 lM. In the same assay, PA were tested and resulted more than twofold more efficacious than PK in activating TRPA1 (Figure 1.17).



PK PA

Figure 1.16 The structure of perillaketone (PK) and perillaldehyde (PA).

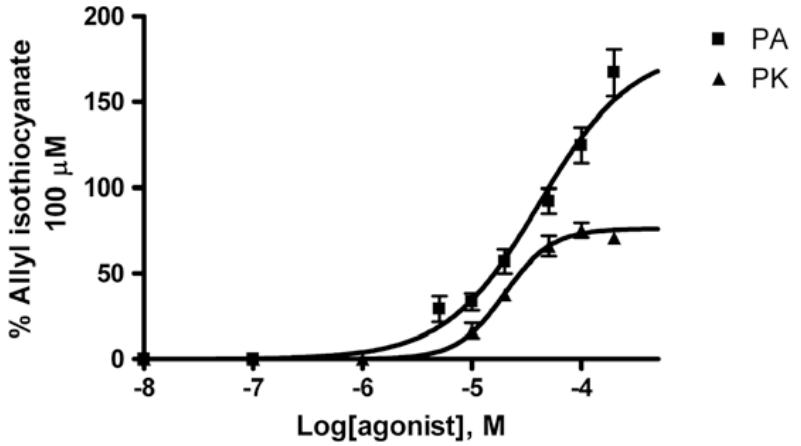


Figure 1.17. Dose-related effects of PA and PK on $[Ca^{2+}]_i$ in HEK-293 cells stably transfected with the rat recombinant TRPA1 channel. Data are expressed as percentage of the maximal effect observed with MO 100 lM (Bassoli *et al*., 2009).

Remarkably, both compounds have an unsaturated carbonyl group in their structure and this could support the hypothesis that this functional group is an important pharmacophore in generating this bioactivity. In fact, TRPA1 can be activated through an 1,4-addition of nucleophilic cystein groups of the receptor protein to appropriate acceptors such as isothiocyanates and unsaturated carbonyls, as we have seen in the previous chapter.

It is interesting to note that, although the efficacy and potency of PK and, particularly, PA at eliciting elevation of intracellular Ca^{2+} via TRPA1 in HEK293 cells was similar, or even superior, to that of pungent TRPA1 agonists, such as the mustard oil isothiocyanates, or carvacrol, or isovelleral, the pungent product of the fungus *Lactarius vellereus*, and polygodial, isolated from the leaves of water pepper, this does not seem to be sufficient to confer to *P. frutescens* a strong pungent or otherwise 'aggressive' taste similar to that experienced instead with mustard, garlic or oregano. This might be due to several reasons, including: (1) the capability of some of these compounds (e.g. isovelleral, polygodial, carvacrol) to activate other TRP channels involved in heat sensitivity (i.e., TRPV1 and TRPV3), (2) the capability, shown here, of PK and PA to immediately desensitise TRPA1, and also to antagonize the TRPM8 channel; (3) the presence in *P. frutescens* of lower amounts of PK and PA as opposed to the possible higher abundance of other TRPA1 agonists in other plants.

1.3.4 Non volatile compounds

The non volatile compounds comprise tritepenoids, phenolics, flavonoids and glycosides, that can be commonly found in some varieties of perilla, in contrast with the volatile comnponents in essential oil (Yu *et al*., 1997).

The structures of some non-volatile terpenoids and sterols isolated from *P. frutescens* are shown in Figure 1.18. As a non volatile monoterpene, perillic acid (**10**) is well-known as a autooxidation product of the perillaldehyde. Besides the widespread sterols, β-sitosterol (**11**), stigmasterol (**12**) and campesterol (**13**) from both the leaves and seeds, Perilla leaves also contain triterpenoids, ursolic acid (**14**), oleanolic acid (**15**) and tormentic acid (**16**).

In common with other members of the Labiate family, *Perilla* leaves contain a rich mixture of phenolics and cinnamates (Figure 1.19).

Typical of these are cinnamic acid derivatives, rosmarinic acid (**17**), caffeic acid (**18**), and ferulic acid (**19**). As a phenolic, protocatechuic aldehyde (**20**) is also present.

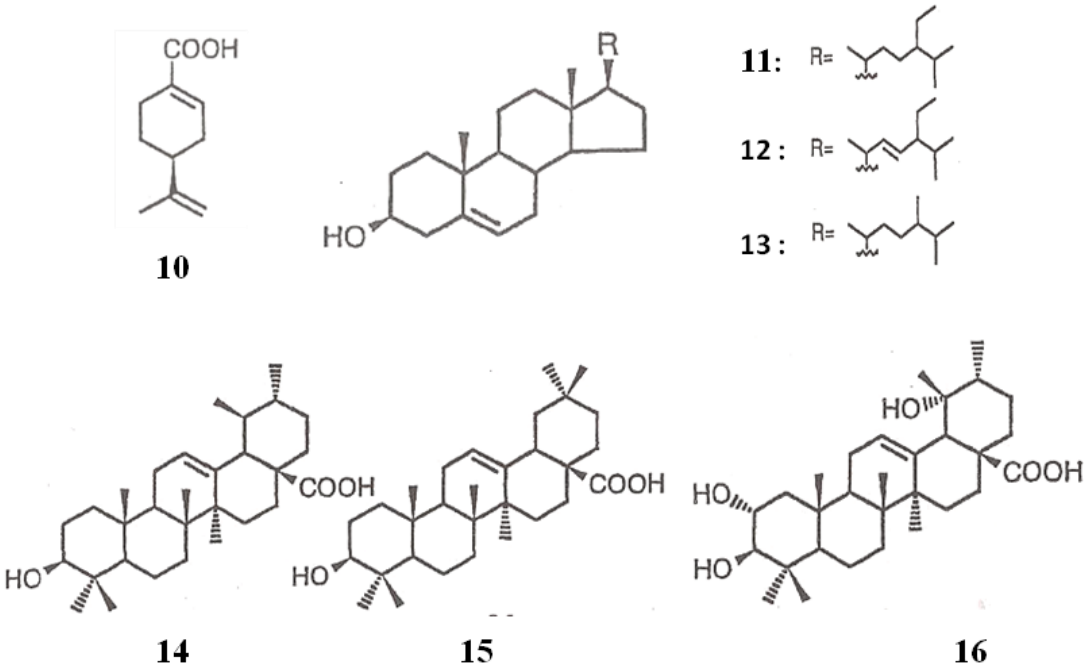


Figure 1.18 Non-volatile terpenoids and sterols isolated from *P. frutescens* (Yu *et al*., 1997).

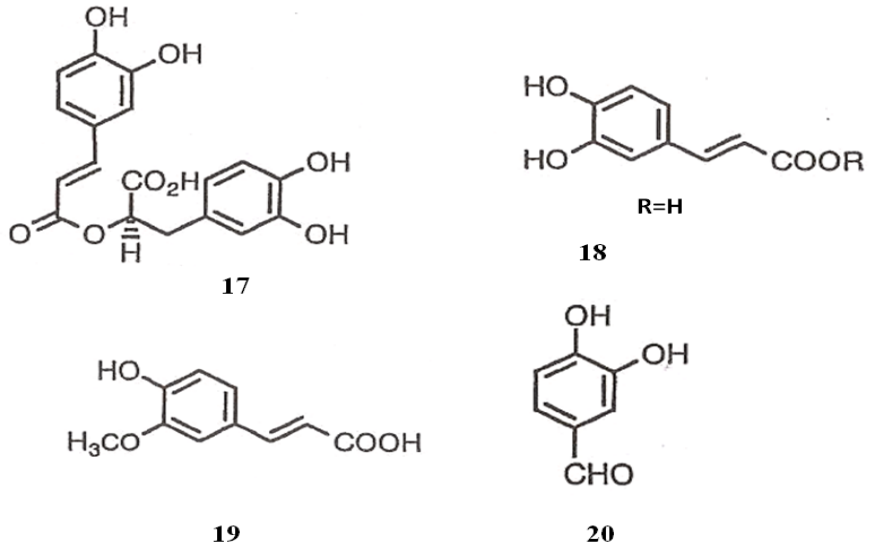


Figure 1.19 Phenolics and cinnamates from *P. frutescens* (Yu *et al*., 1997).

The flavonoids and anthocyanins present in purple perilla are reported in Figure 1.20. Typical flavonoids are apigenin (**21**), luteolin (**22**), scutellarein (**23**), and their glycosides, while typical anthocyanins are acylated glucosides of cyaniding, malonylshisonin (**24**) and shisonin. About twenty glycosides have been found from green and purple perilla leaves. Four monoterpene glucosides perillosides A-D (**25-28**) (Figure 1.21) have been isolated from the methanolic extract of green perilla leaves including perillaldehyde as a major component in the essential oil. Among these, perillosides A and C were found to be inhibitors of aldose reductase, involved in diabetic complications (Yu *et al*., 1997).

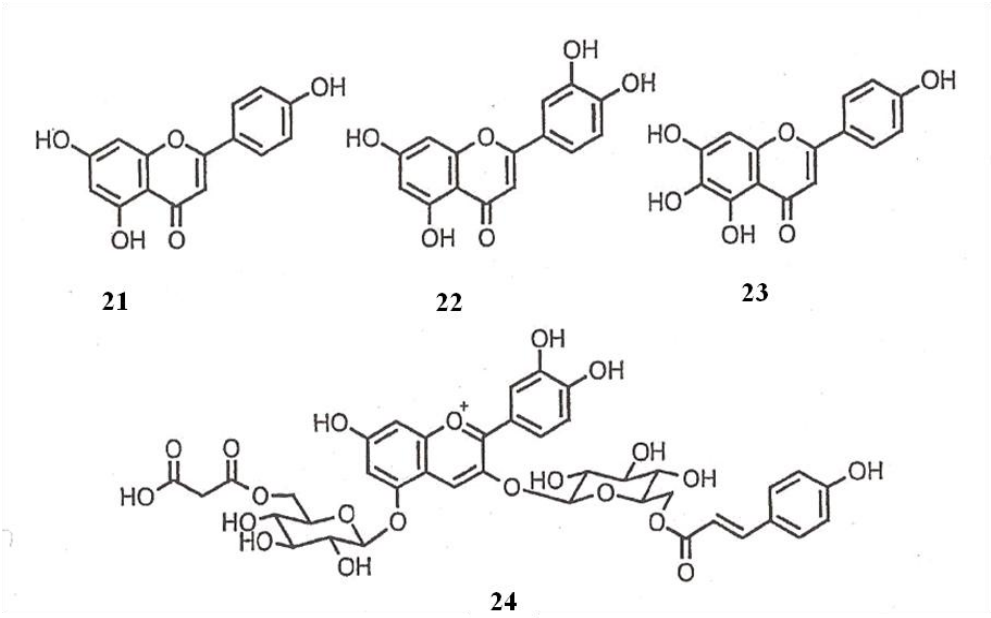


Figure 1.20 Typical flavonoids and anthocyanin from *P. frutescens* (Yu *et al*., 1997).

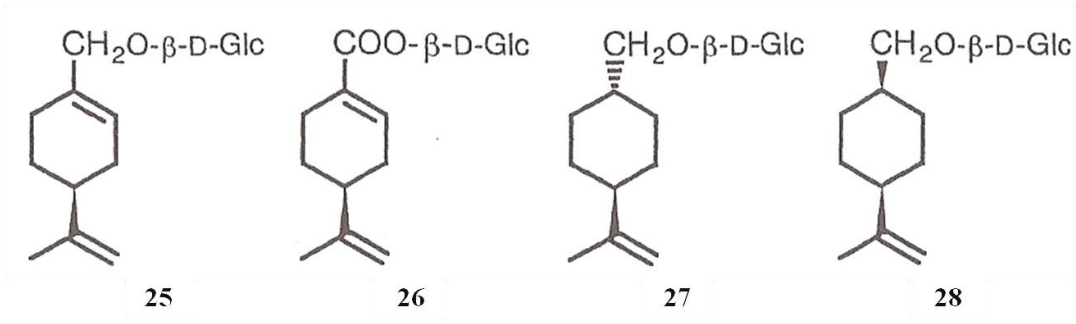


Figure 1.21 Monoterpenoids glucosides from *P. frutescens* (Yu *et al*., 1997).

1.3.5 Uses of *Perilla*

Perilla frutescens (L.) Britton is used in several different ways: seeds are used as food for birds, animals or human consumption; the seed oil is used as a fuel, a drying oil, or cooking oil; the leaves are used as pot-herb, for medicine or for food colouring; and the foliage is distilled to produce an essential oil for flavoring (Pandey *et al*., 2008).

In Japan, the seeds are roasted and then powdered for use in traditional foods as flavoring (Figure 1.22). In China, these seeds are used for oil pressing or they are also crushed and added to cooking for flavoring.

The perilla seeds contain about 45 % oil and most of which (up to 92 %) is composed of unsaturated fatty acids, especially α-linolenic acid (about 68 %) and linoleic acid (about 14 %). Perilla oil also contains 17 amino acids and does not include unhealthy components such as sinapic acid that is rich in rape oil. It is therefore a high quality edible oil and also has wide applications in industry (Hou *et al*., 2005). In the past, the seed oil was also used in the manufacture of lacquer and paper umbrellas.

In Korea, fresh leaves of var. *frutescens* are used for wrapping meats and boiled rice and for pickles (Figure 1.23). Var. *frutescens* is also used for tea, in fact perilla tea is listed on the menu of Korean coffee shops.

In Japan, leaves of var. *crispa* are widely sold in markets and used for tempura and as a garnish with raw fish or noodles. The red leaf type is more valuable and is used for coloring pickles. The red color of pickled plum (umeboshi in Japanese) results from the reaction of the citric acid of the plum with the anthocyanine of var. *crispa* (Figure 1.24) (Nitta *et al*., 2003).

Figure 1.22 Sticky rice ball with seed powder of *P. frutescens* var *frutescens* in Japan (Nitta *et al*., 2003).

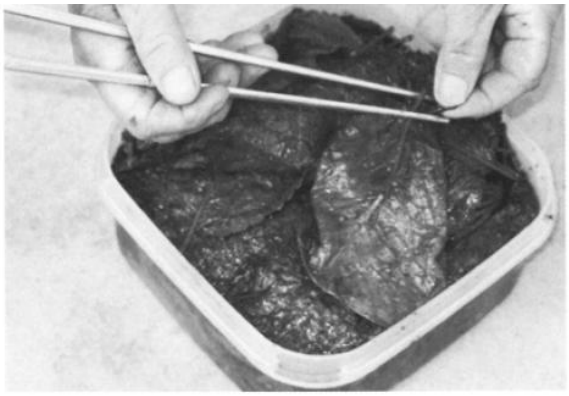


Figure 1.23 Pickled leaves of *P. frutescens* var *frutescens* in Korea (Nitta *et al*., 2003).

Figure 1.24 Pickled plum colored by red leaves of *Perilla frutescens* var. *crispa* in Japan (Nitta *et al*., 2003).

1.3.6 Medicinal properties

Perilla frutescens has traditionally been credited with a long list of medicinal uses because of its biologically active compounds.

In the recently published Chinese Pharmacopoeia, perilla leaf, stem and seed were separately listed as traditional Chinese medicine for different purposes and a number of prescriptions containing perilla leaf, steam or seed as one the ingredients were collected.

The stalks of the plant are traditionally used as an analgesic and anti-abortive agent.

The leaves are said to be helpful for asthma, colds and flus, and to regulate stomach function, while the seeds are employed for dyspnea and cough relief, phlegm elimination, and the bowel relaxation. Recently, animal studies hint that *P. frutescens* might also be useful for a different type of allergy: the severe, rapid reaction known as anaphylaxis, commonly associated with shellfish, peanut, and bee-sting allergies (Lin *et al*., 2010).

Increasingly considerable attention has been given to the anti-allergic, antiinflammatory, antioxidant and anti-tumor promoting substances contained in perilla plants (Makino *et al*., 2003; Banno *et al*., 2004; Peng *et al*., 2005; Gu *et al*., 2009; Meng 2009; Yuri *et al*., 2004).

The main polyphenolic compounds, such as anthocyanidin, luteolin, apigenin, catechin and rosmarinic acid, had been isolated and identified from red perilla and green perilla. It had been reported that the aqueous extract of *P. frutescens* leaves possess a hepatoprotective capacity against t-BHP-induced hepatic and oxidative damage in the rat liver through scavenging reactive oxygen species (ROS) and attenuating the loss of glutathione (GSH). Rosmarinic acid, as one of major polyphenolic compounds in the perilla extract, could reduce lipopolysaccahride-induced and D-galactosamine-induced liver injury. It was reported that luteolin and apigenin function as monoamine transporter activators, which would improve several hypermonoaminergic neuropsychological disorders, especially cocaine dependence, through up-regulating monoamine transporter activity. In particular, luteolin isolated from *P. frutescens* was relatively specific inhibitors of beta-secretase and also could inhibit the nitric oxide (NO) production in lipopolysaccharide-activated microglia in a dose-dependent manner (Feng *et al*., 2011).

2 THE AIM OF THE RESEARCH

Plant diseases due to fungal and bacterial pathogens, and various pests, including insects, nematodes and also weeds, cause severe losses of agricultural and horticultural crops every year. These losses can result in reduced food supplies, poorer-quality agricultural products, economic hardship for growers and processors, and, ultimately, higher prices. Current practices for controlling plant diseases are based largely on synthetic chemicals, but abuse in their employment has favored the development of pathogens resistant to agrochemicals. For many diseases, traditional chemical control methods are not always economical nor are they effective, and fumigation as well as other chemical control methods may have unwanted health, safety and environmental risks (Monte, 2001). Natural products are an excellent alternative to synthetic pesticides as a means to reduce negative impacts to human health and the environment. The move toward green chemistry processes and the continuing need for developing new crop protection tools with novel modes of action, makes discovery and commercialization of natural products as green pesticides, an attractive and profitable pursuit that is commanding attention (Koul *et al*., 2008). Natural plant protectants have many advantages over synthetic pesticides which include:

i. low mammalian toxicity, less hazards to non-target organisms and environmental pollution;

ii. no risk of developing pest resistance to these products, when used in natural forms;

iii. no adverse effect on plant growth, seed viability and cooking quality of the grains (Prakash *et al*., 2008).

It is known that plants produce a great number of secondary metabolites with antimicrobial and biocidal properties. Among the natural compounds with the ability to inhibit the growth of many pathogens, there are: allylisothiocyanate from mustard (*Brassica nigra*), allicin from garlic (*Allium sativum*), eugenol from cloves (*Syzygium aromaticum*), carvacrol from oregano (*Origanum vulgare*), thymol from thyme (*Thymus vulgaris*) and cinnamaldehyde from cinnamon (*Cinnamomum zeylanicum*). Moreover, allylisothiocyanate (typical also of wasabi and horse radish) and allicin have shown insecticidal and antihelmintic activities. A feature of these phytochemicals is that they act

at molecular level by activating the TRPA1 ion channel. TRPA1 is resposible of the chemoesthetic properties of these food plants, which are perceived as a mild pungent or irritating stimulus at gustatory level. The *in vitro* activation of TRPA1 receptor by two of major secondary metabolites contained in the food plant *Perilla frutescens*, perillaldehyde (PA) and perillaketone (PK), is an interesting finding that can have potential agronomical applications.

The antibacterial activity of perilla leaves containing perillaldehyde is documented. Kang *et al*., (1992) demonstrated that PA exhibited a synergistic effect with polygodial against many Gram-positive and Gram-negative bacteria and fungi like *Mucor mucedo*, *Penicillium chrysogenum,* at the minimum inhibition concentrations (MIC) ranging from 31.2 to 1000 µg mL^{-1}. Perillaldehyde showed antibacterial activities on some foodborne pathogens such as *Escherichia coli*, *Listeria monocytogenes*, *Salmonella typhymurium*, *Vibrio vulnificus* (Kim *et al*., 1995) and *Micrococcus luteus* (Friedman *et al*., 2006). The antibacterial ability of leaves extracts from *Perilla frutescens var*. *acuta* was reported by Choi *et al*., (2010) against *Pseudomonas aeruginosa* and by Kim *et al*., (2011) towards *Staphylococcus aureus*. McGeady *et al*., (2002) reported that PA interfered with the morphological change of *Candida albicans* from the relatively benign, cellular yeast form to the pathogenic, filamentous form *in vitro*. Smid *et al*., (1995) described a strong antifungal activity of pure perillaldehyde on *Penicillium hirsutum*, the principal agent of penicillium rot on flower bulbs. Pure PA was also evaluated against three postharvest pathogenic fungi: *Penicillium digitatum*, *P. italicum,* and *P. ulaiense* (Scora *et al*., 1998). While Sekine *et al*., (2007) reported a weak mycelial growth inhibition for Shiso green and Shiso red against *Fusarium oxysporum*.

Furthermore, the activity of perilla seed and/or seed oils as nematicidal and insect repellent was also reported (Taylor, 2003). In this patent the efficacy of the plant was ascribed to the high amount of linoleic acid, α- linoleic acid, oleic acid and other unsaturated fatty acids that should act as dehydrating agents which stop the feeding and reproductive processes of the nematodes.The nematicidal activity of *P. frutescens* essential oil was demonstrated against the pinewood nematode *Bursaphelenchus xylophilus* (Choi, 2007); in this paper a commercial sample of perilla from the Korean market was used and a chemical characterisation of the components was not done. The essential oil from *P. frutescens* (L) Britton var. *orientalis* was also reported as weakly active against the rice weevil, *Sitophus oryza*e (Lee, 2001) and the authors attributed the activity to the perillaldehyde contained in the mixture. Perillaldehyde and other monoterpenoids biosynthesised by perilla plants

showed a control activity against the saprophytic nematode *Caenorhabditis elegans* and the root lesioning nematodes *Pratylenchus penetrans* (Tsao, 2000). PA showed a good activity also against the fish parasitic nematode *Anisakis* spp. and the dog roundworm *Toxocara canis* (Goto, 1995).

Although the antimicrobial activity of the perilla extracts containing PA as main compound is described, no earlier studies are reported in the literature with regard to the antibacterial and antifungal activity of perillaketone. Moreover, the nematicidal activity of pure perillaketone has never been evaluated.

The aim of this work is to determine the antimicrobial activity of the crude extracts and of essential oils obtained from the leaves of two varieties of *Perilla frutescens* grown in Northern Italy (named PA-type and PK-type respectively). Commercial perillaldehyde and synthetic perillaketone were also assayed against some important phytopathogenic bacteria and fungi. In addition, the nematicidal efficacy of the pure perillaketone isolated from the leaves of cultivar PK-type, was also evaluated.

The study focuses mainly on the following objectives:

- ✓ Steam distillation, organic solvent extraction, purification and synthesis of active compounds from two cultivars of *Perilla frutescens*, optimizing the procedure to obtain the needed material for the biological assays.
- ✓ Realization of different *in vitro* tests on several plant pathogenic bacteria and fungi belonging to different *phyla*, to identify the most sensitive target.
- ✓ Realization of *in vivo* tests in greenhouse to evaluate the potential preventive activity of assayed compounds against *Sphaerotheca fuliginea* on *Cucumis sativus*.
- ✓ Evaluation of nematicidal activity of perillaketone against 2nd instar larvae juveniles of cyst nematode *Heterodera daverti* Wouts *et* Sturhan.

3 MATERIALS AND METHODS

3.1 Chemical procedures

Reagents were of commercial grade purity and the solvents were dried with standard procedures.
Chromatography was carried out on 220-240 mesh silica gel using the flash methodology (Still, 1978).
Thin-layer chromatography was obtained on Merk precoated silica gel 60 F_{254} plates and the spots were visualised by UV at 254 nm.
HPLC analyses were recorded on a Varian SD 200 liquid chromatograph.
NMR spectra were recorded on Bruker AMX-300, using tetramethylsilane (TMS) as internal standard; coupling constant (*J*) are given in Hertz.

Abbreviation: AcOEt ethyl acetate, DCM dichloromethane, DMSO dimethylsulfoxide, MeOH methanol, THF tetrahydrofuran.

3.1.1 Crude extracts

Frozen leaves (240 g) of adult plants were cut into small pieces and were extracted with DCM for 24 h at room temperature. The extraction with mechanical stirring was repeated for two times. The resultant extracts were combined and concentrated under reduced pressure to give green PA-Ex and PK-Ex weighing 3.2 g and 3.6 g, respectively.
The two samples were analyzed by HPLC and NMR to confirm the chemotype classification.

3.1.2 Essential oils

Separate aliquots of 20.0 g of both cultivars were steam distilled for 1 hour and 30 minutes to obtain 200 mL of aromatic water. This solution was extracted three times with 100 mL of DCM, then was dried over anhydrous sodium sulfate, filtered and the solvent evaporated. PA-EO (34.7 mg) and PK-EO (65.5 mg) were preserved in a sealed vial at 4 ±2°C until further analysis.

3.1.3 Chemotype classification

Principal secondary metabolites of *Perilla frutescens* samples were identified and analyzed by TLC, HPLC and NMR. Thin-layer chromatography was performed on Merck precoated silica gel 60 F254 plates and the spots were visualised by UV at 254 nm. HPLC analyses were carried out using a Varian SD 200 liquid chromatograph with RP column Alltima C18 (250 mm lenghth, 4-6 mm ID, 5µ, Alltech) equipped with a UV detector at 254 nm.

20 µL of the test solutions at concentration of 1mg mL^{-1} were injected, and the column was eluted with a linear gradient with a mobile phase containing solvent A (water) and solvent B (methanol). The solvent gradient was programmed from 70% to 100% B in 45 min with a flow rate of 1.0 mL/min. The crude extracts were filtered on RP 18 and through a cellulose acetate membrane filter of 0.45 mm, prior to HPLC analysis. Data acquisition was done with the Galaxy software of Varian. The identification of predominant compounds in the test samples was based on comparison of their relative retention times with those of pure perillaldehyde, as commercial preparation with at a nominal purity of least 92% and perillaketone, obtained previously by synthesis. In mentioned conditions the two standards have retention times of 10.28 min (PK) and 13.16 min (PA). The samples from *Perilla frutescens* var. crispa contained perillaldehyde as the main secondary metabolite and thus were classified as PA-chemotype. The samples from Korean cultivar included, instead, almost exclusively perillaketone and were classified as PK-chemotype. PA was present at 65.1% in the extract and at 95.8 % in the essential oil. Whereas PK was contained at 79.8% in the extract and at 99.0% in the oil. The HPLC profiles of the essential oils fom the two cultivars are shown in Figure 3.1 and 3.2. The figure 3.3 and 3.4 reported the ^{1}H-NMR spectra of these oils.

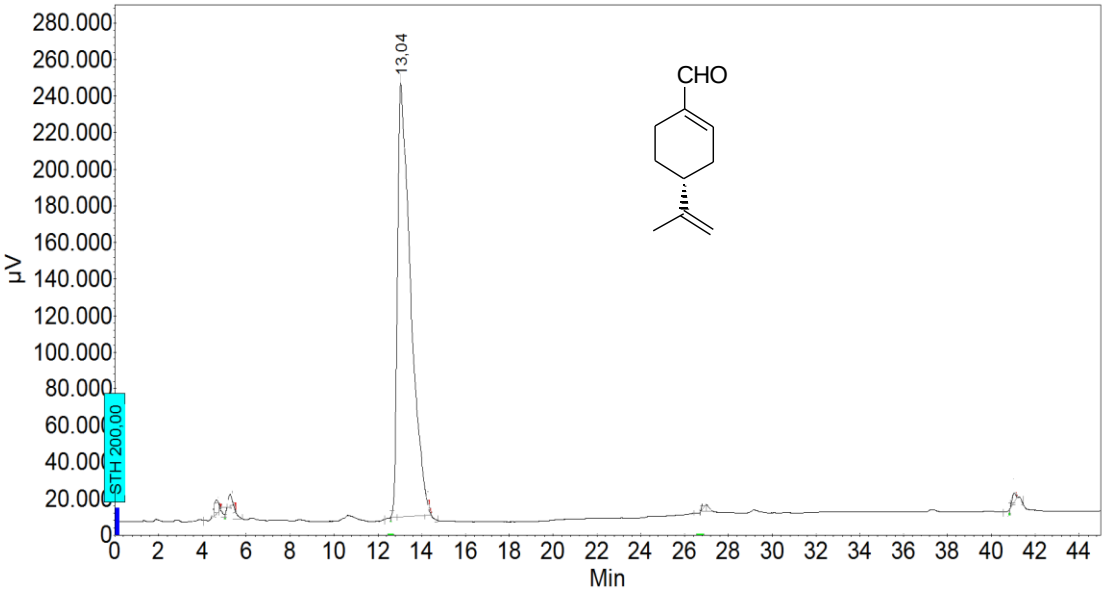


Figure 3.1 Chromatogram of essential oil PA-type.

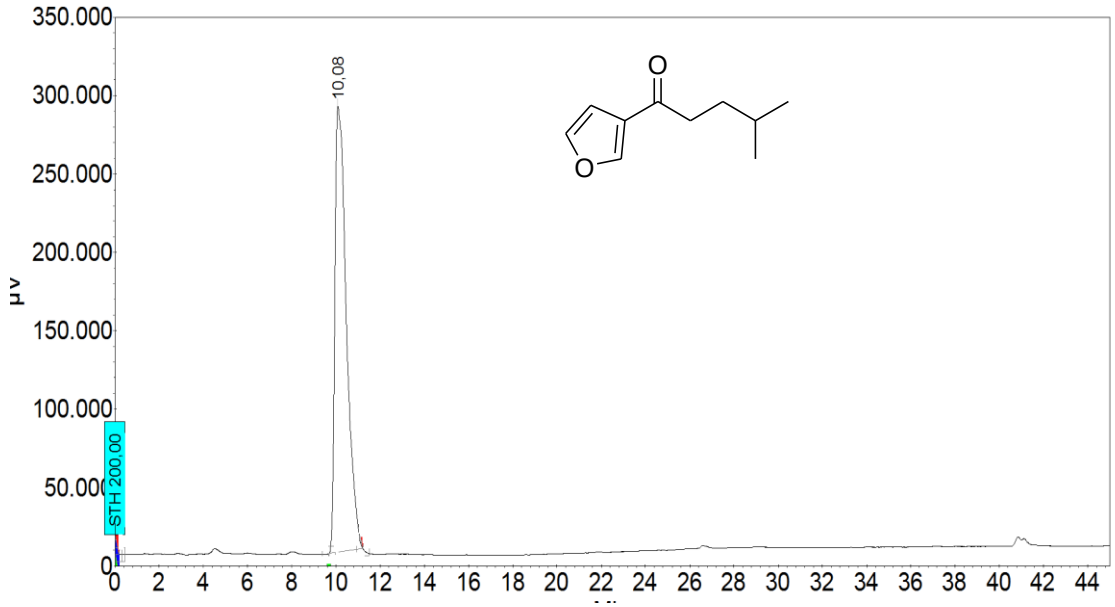


Figure 3.2 Chromatogram of essential oil PK-type.

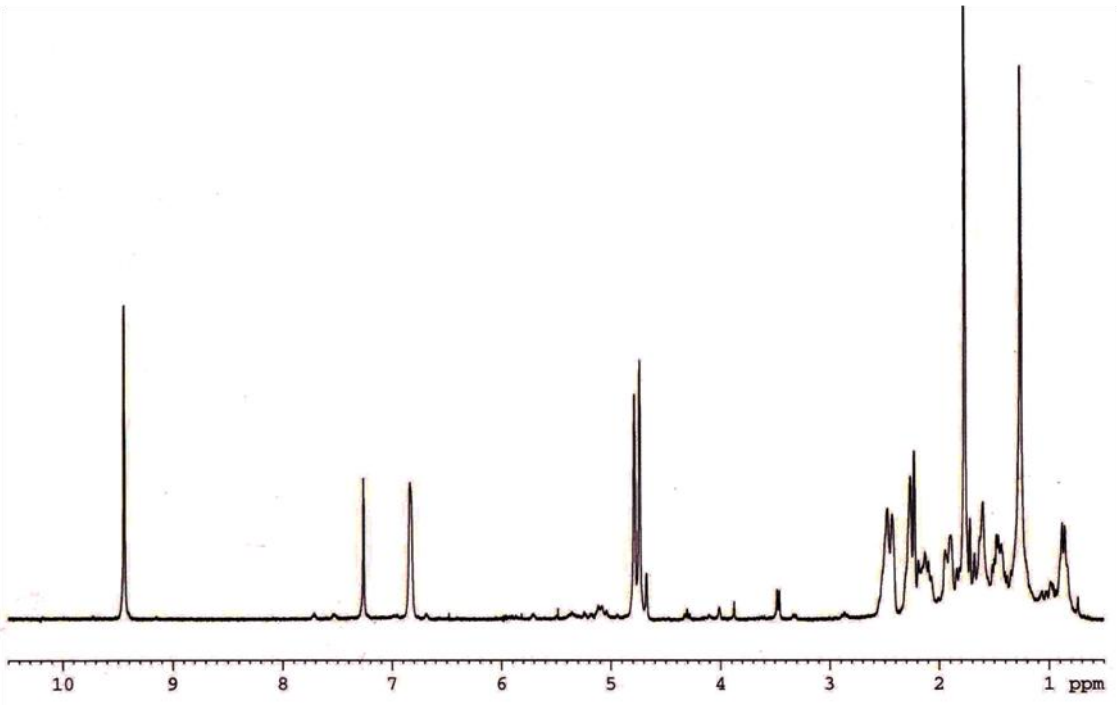


Figure 3.3 ^{1}H-NMR ($CDCl_3$) spectrum of of essential oil PA-type.

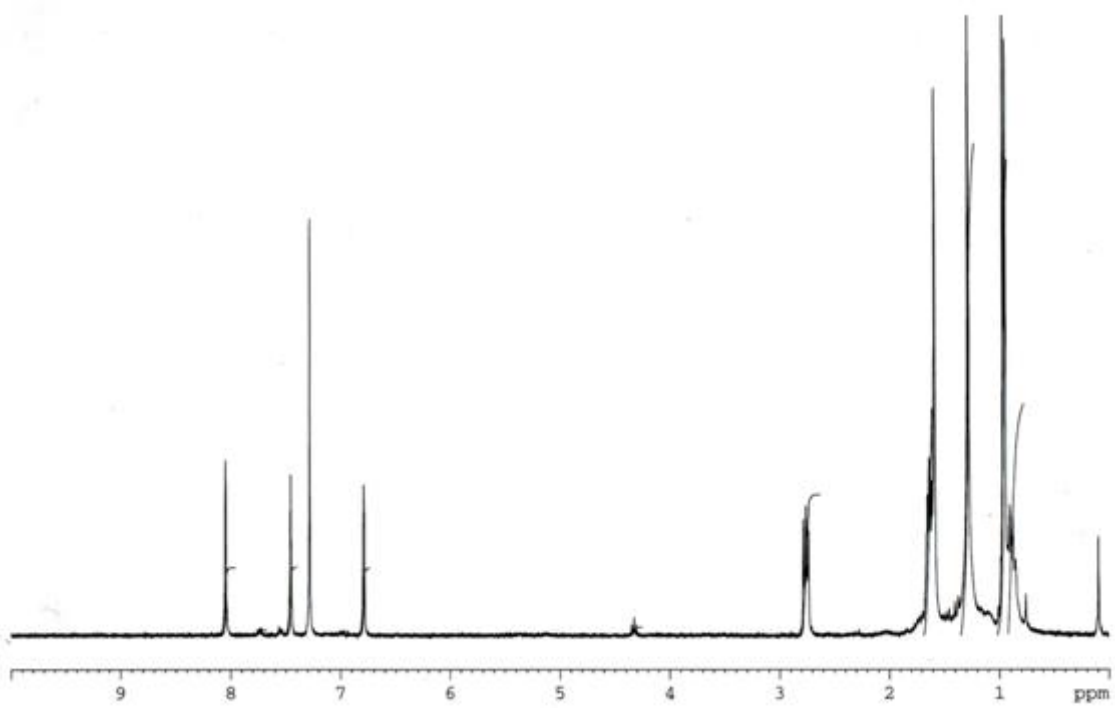
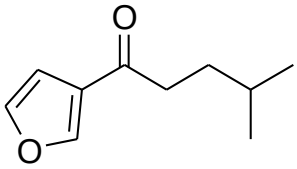


Figure 3.4 ^{1}H-NMR ($CDCl_3$) spectrum of of essential oil PK-type.

3.1.4 Synthesis of Perillaketone (*1-furan-3yl-4-methyl-pentan-1-one)*, PK (34)



The Grignard addition on fresh distilled 3-furaldehyde gives a mixture of alcohol **33** (19%) and perillaketone **34** (12%).

Under a nitrogen atmosphere, 264 mg (11 mmol) of Mg in dry THF (5.5 ml) were added a catalytic amount of I_2 and a solution of fresh distillate 3-methyl-butyl-bromide (1.66 g, 11 mmol) in 5.5 ml of dry THF. The Grignard reagent was added to a solution of 3-furaldeyde (1.00 g, 11 mmol) in10 ml of dry THF and the solution stirred at room temperature for 24 h. The reaction mixture was acidified with satured aqueous ammonium chloride solution, concentrate in vacuum and extracted with DCM. After drying of the combined organic layer with Na_2SO_4 and evaporation of solvent under vacuum, the product was isolated by flash chromatography (petroleum ether/diethyl ether 85/15) Was obtain as first fraction 205 mg (12%) of pure PK **34** as colourless oil and as a second fraction the alchol **33** 314 mg (19%).

^{1}H NMR ($CDCl_3$) δ: 0.93 (6H, d, J=6.43 Hz, 2 CH_3), 1.61 (3H, m, CH_2CH), 2.74 (2H, t, J=7.12 Hz, CH_2CO), 6.76 (1H, dd, J=1.84 e 0.92 Hz, H-4), 7.42 (1H, dd, J=1.84 e 1.47 Hz, H-5), 8.01 (1H, dd , J=1.47 e 0.92 H-2).
^{13}C NMR ($CDCl_3$) δ: 22.2, 27.6, 33,0, 38.2, 108.5, 127.5, 144.0, 146.9, 195.2.
GC-MS, Rt 14.05 min, m/z, (%): 166 (M^+, 5), 110 (90), 95 (100).
HPLC: Rt 10.28 min.

***1-furan-3yl-4-methyl-pentan-1-ol* 33**

^{1}H NMR ($CDCl_3$) δ: 0.85 (6H, d, J=7.02 Hz, 2 CH_3), 1.05-1.85 (5H, m CH_2CH_2CH), 4.62 (1H, t, 6.88, CHOH), 6.38 (1H, bs, H-4), 7.37 (2H, bs, H-2 e H-5).
^{13}C NMR ($CDCl_3$) δ: 22.47, 27.86, 33.62, 35.56, 67.19, 108.34, 129.11, 138.94, 143.23.
HPLC: Rt 12.14 min (99.0% area).

The same product was obtained by reduction with $NaBH_4$ (11.4 mg, 0.3 mmol) in 0.5 ml of ethanol of a solution of PK **34** (50 mg, 0.3 mmol). After stirring at room temperature for 2 h, the mixture was concentrate in vacuum, quenched by addition of satured aqueous ammonium chloride solution and extracted with DCM for several times. The product was purified by flash column chromatography to give 30 mg of colourless oil. (60%).

3.1.5 Extraction of Perillaketone

Perillaketone, for the nematicidal assay, was extracted from grinded freeze-dried leaves (30 g) of the Korean Perilla with hexane four times for 2 hours each at room temperature. The solution was filtered, dried with Na_2SO_4 and the solvent evaporated. The crude product obtained (263 mg) was purified by flash chromatography (hexane/ether 8.5/1.5) to give perillaketone as a pure colorless oil (16 mg).
The purity (93%) was checked by HPLC, with the same analytical protocol reported before.
GC–MS analyses were performed on a Shimadzu GC–MS spectrophotometer QP5000 with helium carrier at a flow of 0.8 mL/min, and with polymethyl siloxane at low polarity AT-1ms (30 m of length, 0.25 mm id, Alltech) as capillary column-bonded phase; temperature program: 50°C for 5 min, rate 5°/min to 240°C in 40 min, 240°C for 15 min; injection in splitless mode, injector temperature 220°C, interface temperature 300°C; acquisition range: 50–450 *m*/*z*; detector condition 70 eV; current 60 μA; electromultiplicator: 1300V. The Class 5K software of Shimadzu was used for data processing.

3.2 Antimicrobial assays

The crude extracts and the essential oils of both varieties (PA and PK types), commercial PA and PK obtained by synthesis, were tested against a panel of selected microorganisms by *in vitro* and *in vivo* assays.

3.2.1 Microorganisms

The antimicrobial activity of tested compounds was valued on phytopathogenic bacteria and fungi, listed below:

Arthrobacter ilicis IPV 2504, *Bacillus subtilis* IPV 2430, *Clavibacter michiganense* pv *insidiosum* IPV 2487, *Clavibacter michiganense* pv *nebraskense* IPV 2510, *Clavibacter tritici* IPV 2507, *Curtobacterium flaccumfaciens* IPV 2485, *Curtobacterium flaccumfaciens* subsp *betae* IPV 2483, *Curtobacterium flaccumfaciens* subsp *oortii* IPV 2513, *Curtobacterium flccumfaciens* subsp *poinsettiae* IPV 2502 (Gram +); *Pseudomonas syringae* pv *mors-prunorum* IPV 2605, *Xanthomonas anoxopodis* pv *dieffembachiae* IPV 2620, *Xanthomonas anoxopodis* pv *poinsetticola* IPV 2626, *Xanthomonas anoxopodis* pv *vignicola* IPV 2625, *Xanthomonas vesicatoria* IPV 2615 (Gram -); *Alternaria alternata* RF8, *Aspergillus niger* IPV-F303, *Armillaria mellea* (strains: 3A, 7A, 4B, 5B, 12B, 4C, 6C, 12C, 5D, 12D), *Bipolaris oryzae*, *Botrytis cinerea* F4-11, *Cercospora beticola*, *Cladosporium cladosporioides* IPV-F167, *C. cladosporioides* CBS 574.78 A, *C. cucumerinum*, *Colletotrichum dematium* f sp *circinans* X, *Fusarium oxysporum* f sp *lactucae* L55, *Fusarium roseum*, *Penicillium corylophilum* L2, *Pyrenophora graminea*, *Pyricularia oryzae* BA43, *Rhizoctonia solani*, *Sclerotinia sclerotiorum.*

The yeast *Saccharomyces cerevisiae* CBS 1171was also used.

The strains were obtained from the culture collection of Department of Agri-Food and Urban System Protection and Biodiversity Enhancement (DiPSA), Faculty of Agriculture in Milan.

The freeze-dried bacterial strains were reconstituted by adding nutrient broth.

The samples were then incubated for 5 days at 24 ±2°C. Fungal cultures were maintained on different substrates and stored at +4±2°C.

3.2.2 Culture media

In the present study different growth media were used, their composition is reported below:

- **Potato Dextrose Agar** (PDA, Oxoid)

39 g of powdered medium to dissolve in 1 L deionized water, containing:

Potato Extract	4.0 g
Glucose	20.0 g
Agar	15.0 g

Final pH 6.5 ± 0.2

- **Malt Agar** (MA, Difco)

45 g of the medium to dissolve in 1 L of purified water, containing:

Malt Extract	30.0 g
Agar	15.0 g

Final pH 6.5 ± 0.2

- **Nutrient Agar** (NA, Difco)

23 g of powdered medium to dissolve in 1 L deionized water, containing:

Beef Extract	3.0 g
Peptomeat	5.0 g
Agar	15.0 g

Final pH 7.0 ± 0.2

- **Nutrient Broth** (NB, Difco)

8 g of powdered medium to dissolve in 1 L deionized water, containing:

Bacto Beef extract	3.0 g
Bacto Peptomeat	5.0 g

Final pH 7.0 ± 0.2

- **Czapek Dox Broth** (CDB, Difco)

35 g of powdered medium to dissolve in 1 L deionized water, containing:

Saccharose	30.0 g
Sodium Nitrate	3.0 g
Dipotassium Phoshate	1.0 g
Magnesium Sulfate	0.5 g
Potassium Chloride	0.5 g
Ferrous Sulfate	0.01 g

Final pH 6.5 ± 0.2

- **Medium to inhibit rhizomorph formation** (ARM) (Anselmi and Govi, 1996)

Glucose	10.0 g
L-asparagine	2.0 g
KH_2PO_4	1.75 g
$MgSO_4$	0.75 g
Agar	10.0 g
Vitamin B_1	1.0 g
Deionized water	1 L

Final pH 6.5 ± 0.2

The culture media were heated to boiling to dissolve completely. The media were adjusted to the desired pH by adding NaOH or HCl (1 N), autoclaved at 121°C, 1 atm, for 20 minutes and then cooled to 50°C.

3.2.3 *In vitro* assays

Following assays were employed for the determination of antimicrobial potential of tested compounds.

Disk diffusion method

The antimicrobial activity was determined by paper disk diffusion assays on microorganisms including strains of phytopathogenic fungi (*A. mellea*, *A. niger* F303, *B. cinerea* F411, *C. cladosporioides* IPV- F167, *F. roseum*, *P. corylophilum* L2) and mentioned bacteria. *S. cerevisiae* 1171 was also tested. Microorganisms were grown in Petri dishes (6 cm diameter) containing 10 mL PDA for fungi, NA for bacteria and ARM for *A. mellea*. 200 µL of spore suspension (10^6 spores/mL) of *F. roseum*, *B. cinerea* F4-11 and *C. cladosporioides* IPV- F167 was spread over the surface of each agar plate with a sterile, bent glass rod. For the other microorganisms 1 mL of the spore suspension (10^7 spores/mL) was inoculated in 150 mL of the appropriate medium. In the case of *A. mellea*, a mycelium disc of 6 mm diameter, cut out from the edge of a growing colony, was placed upside down about 1cm from the edge of a Petri dish of ARM. PA-EO, PK-Ex and PK obtained by chemical synthesis, were dissolved in MeOH to give concentrations of 250, 500, 1000, 2000 µg mL^{-1} and 20 µL of these solutions were used to wet sterile paper disks (Schleicher & Schuell, 6 mm diameter) put in the centre of inoculated plates or opposite the inoculum in the case of *A. mellea*. Disks containing only solvent were used as negative control. Inoculated Petri dishes were incubated at 20°C (*A. mellea*) or 24°C (other microorganisms) for 3-7 days. Incubation was extended to 1 month in the case of *A. mellea*. Antimicrobial activity was assessed by measuring the diameter of the inhibition zone around the disks.

Poisoned food technique

Fungitoxicity was also studied following a poisoned food technique which produces hyphal growth inhibition. Samples were dissolved in dimethyl sulfoxide (DMSO) and required amounts of the solutions were added to sterile cool MA to give concentrations of 100, 1000, 2000 µg mL^{-1} in the case of the crude extracts and of 100 µg mL^{-1} for pure

PA. For the evaluation of the essential oils, the tested concentrations were 100, 200, 300, 400 and 500 µg mL^{-1} in the case of *Cladosporium* spp, and of 200 and 500 µg mL^{-1} for the other fungi. Amended media were inoculated with mycelia plug (7 mm) cut from the edge of actively growing colonies and placed upside down in the center of dishes. Then, the plates were scaled with parafilm and incubated at 24°C±2°C. The radial growth of the colony was measured 3, 5 and 7 days after incubation. MA plates containing DMSO, without samples, and the compound solutions were used as negative control. The effect of the natural compounds were compared with that of standard fungicides: azoxystrobin (1 µg mL^{-1}) against *P. teres* and *P. oryzae*; cyprodinil (1 µg mL^{-1}) towards *B. cinerea* and *A. alternata;* and prochloraz (0.1 µg mL^{-1}) for the other fungi.

Antifungal activity, expressed in terms of percentage of mycelial growth inhibition, was calculated using the following equation:

$$\% \text{ Mycelia inhibition} = \frac{(C-T)}{C} \times 100$$

where, C is the mycelia diameter of the control and T is the mycelia diameter of treated samples.

All tests were carried out in triplicate and inhibition percentages were reported as means ±SE of triplicates.

To determine the nature of toxicity (fungistatic/fungicidal) of each sample, the mycelial discs totally inhibited by the compound, were transferred on fresh medium. The treatment was considered fungistatic if the growth of the fungus restarted and fungicidal if not.

Bioautography on thin-layer plates

10 µL of test compounds (PA-Ex, PK-Ex, PA-EO, PK-EO, PK obtained by chemical synthesis and commercial PA) dissolved in EtOAc were applied as small spots on 6,5x16,8 cm thin-layer Plates TLC (Si 60; 0.25 mm; Merck) to give a final concentration of 1, 10, 20, 50, 100 µg/application zone. Prochloraz was used as positive control at 10^{-3}, 10^{-2}, 10^{-1}, 1, 10 µg/application zone.

The organic solvent was evaporated by a stream of air. The TLC plates were homogeneously sprayed with spores of *C. cladosporioides* IPV-F167 suspended in

Czapek Dox *Broth* (10^6 spores/mL). Plates were incubated for 5 days in a moist chamber at 24±2°C in the dark. Fungal growth inhibition appeared as clear zones against a dark background, indicating the minimum amount of compounds required for it.

Spore germination assay

Both essential oils and commercial PA were checked for the suppression on the germination of *C. cladosporioides* IPV-F167 spores. For this purpose, spore suspensions in deionised water added by 0.01% Tween 20 adjusted to approximately 10^6 spores/mL by using KOVA haemocytometer (Hycor Biomedical Inc. USA). Petri dishes containing MA amended with PA and PK-oils at 100, 250 and 500 µg mL^{-1}, and commercial PA at 100 µg mL^{-1} were inoculated by 20 µL of spore suspension. The plates in duplicate were incubated at 20±2°C for 24 hrs and then examined under a light microscope for spore germination. DMSO as a negative control at 500 µg mL^{-1} did not inhibit the spore germination of tested pathogen. Percentage spore germination was calculated by observing 100 spores for each recurrence, according to the following formula:

$$\% \text{ Spore germination} = \frac{\text{Germinated spores (No.)}}{\text{Total spores (No.)}}$$

3.2.4 *In vivo* assays

Preventive activity of the tested compounds were evaluated by *in vivo* assay on *Sphaerotheca fuliginea/Cucumis sativus L.* (cucumber powdery mildew). *Cucumis sativus* L. cv "Lungo della Cina" used in this study was grown in plastic pots (diameter 11 cm) with artificial light (14 h per days) at 22 ± 2° C and 70 ± 20% RH. Cucumber plants were employed when the first true leaf was fully developed. Plants inoculation was performed spraying on both the leaf surfaces the *S. fuliginea* spore suspension ($1 \cdot 10^5$ spore mL^{-1}) in deionised water plus 0.01% Tween 20, obtained from previously infected host plants. Assayed compounds were dissolved in a mixture of deionised water (80 mL), and acetone (20 mL) with Tween 20 (40 mg) as a surfactant. Crude extracts (100 and

1000 µg mL^{-1}), essential oils (100, 500 and 1000 µg mL^{-1}) and commercial PA (0.1, 1, 10 and 100 µg mL^{-1}) were applied 5 hours before the inoculation on leaf surfaces of five *C. sativus* plants, respectively. A commercial fungicide, metrafenone (0.01 µg mL^{-1}) was also used as positive control.

Disease severity was rated visually on the basis of the percentage of infected area on each leaf, using a 0–5 index recorded according to a six-point linear scale, from 0 to 5 with a 20% interval (0 = no symptoms; 1=0-20%; 2= 20-40%; 3= 40-60%; 4= 60-80%; 5= 80-100%). The compounds activity was calculated as percentage of disease suppression in comparison with the inoculated untreated plants. Observations were made 8 and 10 days after the treatment.

3.2.5 Statistical analysis

Poisoned food experiments were subjected to variance analyses carried out using DSAASTAT for Excel, version 1.101 (available at http://www.unipg.it/~onofri/ DSAASTAT/DSAASTAT.htm; Onofri, 2011). Differences between means were tested through Duncan's test and values with $p< 0.05$ were considered significantly different.

3.3 Nematicidal assay

The assays were realized in collaboration with Department of Agrarian Entomology and Zoology, Federico II University of Naples, on 2nd instar larvae juveniles of cyst nematode *H. daverti*. These active individuals were extracted from roots of carnation plants grown in a ornamentals greenhouse in Torre del Greco (Naples), utilizing cottonwool filter method of Oostenbrink for 24 hours (Oostenbrink, 1960).

The solution for the direct contact test was prepared dissolving 15 mg of perillaketone in 150 µl of alcohol (5% v/v) (A) and then 50 µl of this solution in distilled water (W) up to 1 ml (5 mg/ml) (solvent A+W). The controls were: a positive control treated only with 2 ml of solvent (A+W) (5 ml/ml) and another one with 2 ml of nematicide ethoprophos with contact action (4 ml/l). Moreover a control with 2 ml of pure distilled water (W) was performed to evaluate the possible activity of alcohol (known for its dehydrating action)**.** 100 of 2nd instar larvae juveniles (total 400) were dipped into the sample solutions placed in drop-bottom glass capsules and observed, by stereoscope, after 1 day in the 4 tested solutions and then transferred in distilled water. The specimens considered unmotile or dead were transferred in different glass capsules with distilled water and if, at next observation, they restarted the motility they were placed back with the motile individuals. The unmotile larvae juveniles include a high number of dead distinguishable only in the next observation. They were examined, at irregular time intervals (from 1 to 6 days) for 26 days and their mortality and unmotility were detected. The assays were carried out at a constant temperature of 24°C and in the darkness. The capsules were closed with laboratory film to avoid evaporation.

4 RESULTS AND DISCUSSION

4.1 Plant material

Two different cultivars of *Perilla frutescens* were investigated in this study: crisp green-leaved perilla (Tokita green) and a smooth green-leaved Korean variety (from Kitazawa seed). The plants were seeded and grown in open field at the botanical garden of Fondazione Minoprio, Vertemate con Minoprio (CO) (Martinetti *et al*., 2010). The leaves were harvested at the end of flowering season and immediately frozen. The leaves of each variety were used to prepare the crude extracts and the essential oils for the biological tests.

Chemical analysis of the samples allowed identification of perillaldehyde and perillaketone as the main secondary metabolites of the crisp and the smooth green-leaved perilla respectively, and thus the classifcation in PA and PK- chemotypes. Therefore the activity of organic extract PA and PK-type (PA-Ex and PK-Ex) and the essential oils PA and PK type (PA-EO and PK-EO) were evaluated by *in vitro* and *in vivo* assays.

4.2 Perillaketone: synthesis and extraction

Perillaketone (PK) is no commercially avaible and was obtained by chemical synthesis and isolated from freeze-dried leaves of the Korean perilla.

4.2.1 Synthesis of Perillaketone

For the synthesis of perillaketone several approaches have been described: photochemical isomerisation (Zamojsk *et al*. 1977) of a precursor oxethane with PTSA as catalyst in a protic solvent and the oxidation of furyl alcohol, organometallic reaction with litiofuran as reagent (Brown *et al*. 1987, Bailey *et al*. 1991) or acylation reaction with organomanganese reagents (Cahiez *et al*.1992) are some examples. We improved the synthesis of perillaketone using the classical Grignard reaction starting from commercially available 3-furaldehyde (**32**) and 3-methyl-buthylmagnesiumbromide (**31**)

generated in situ from the corresponding alkyl halide 1 in dry THF (figure 4.1).
The reaction on fresh distilled 3-furaldehyde gives a mixture of alcohol **33** (19%) and perillaketone **34** (12%) after chromatography purification (silica gel and a mixture of petroleum ether/diethyl ether 85/15 as eluent).

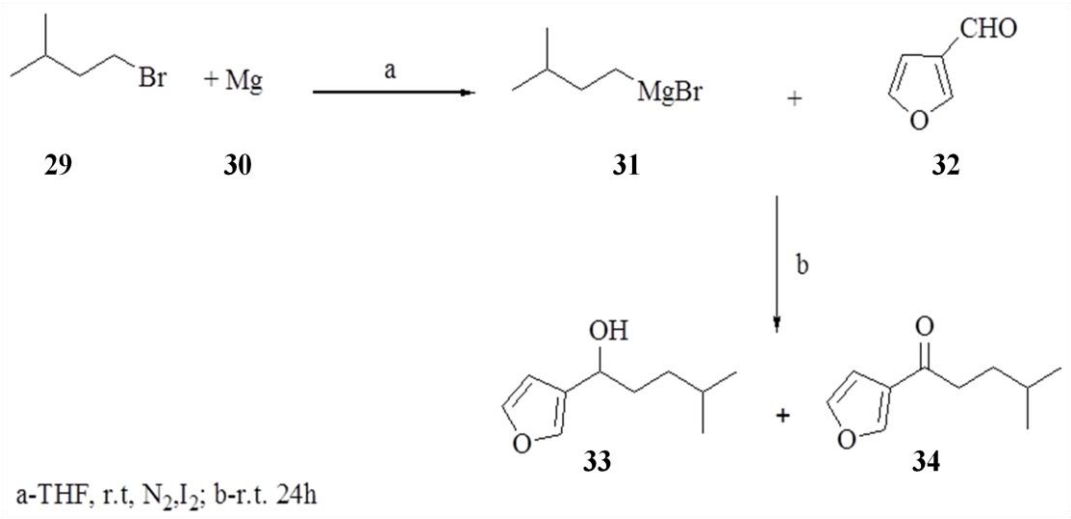


Figure 4.1 Synthesis of Perillaketone.

4.3 Antimicrobial activity of *Perilla frutescens* compounds

The antimicrobial activity of the leaves crude extracts and essential oils from the two varieties of perilla, synthetic perillaldehyde and perillaketone, were determined against a set of common phytopathogenic microorganisms. The antifungal activity of tested compounds was assessed on fungi and bacteria belonging to different *phyla*, causing widespread diseases on a variety of crops.
In the *in vivo* experiments, the efficacy of the natural compounds and commercial PA was evaluated on cucumber powdery mildew (*Sphaerotheca fuliginea*/*Cucumis sativus*). Finally, against some *Cladosporium* spp the antifungal efficacy was examined *in vitro* by disk diffusion method, bioautography on thin-layer plates, spore germination assay and poisoned food technique.

4.3.1 *In vitro* activity

Preliminary assays were carried out by disk diffusion method against selected fungal and bacterial strains, and then by poisoned food technique for the fungi, only.

Disk diffusion assay

The activity of tested compounds was determined on various fungi including ten strains of *Armillaria mellea, A. niger* F303, *B. cinerea* F411, *F. roseum* and *P. corylophilum* L2. Antibacterial efficacy was also evaluated against nine species of Gram-positive bacteria and five species of Gram-negative bacteria. S. *cerevisiae* 1171 was also tested.
PA-EO, PK-Ex and PK obtained by chemical synthesis were assayed at four concentrations: 250, 500, 1000 and 2000 µg mL^{-1}. The antifungal and antibacterial activity of the tested substances resulted generally low. Small inhibition zone was observed for the crude extract PK-type at 2000 µg mL^{-1} against *Xanthomonas vesicatoria* 2615 and *Xanthomonas anoxopodis* pv *vignicola* 2625 (Figure 4.2), only.

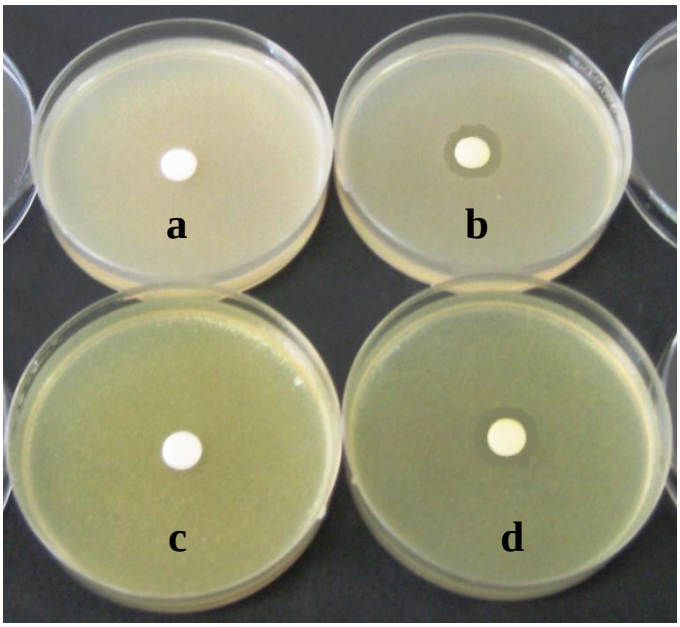


Figure 4.2 Inhibition zone induced by PK-Ex at 2000 µg mL^{-1}.
On the top: *Xanthomonas vesicatoria* 2615 (a: control, b: tested compound).
On the bottom: *Xanthomonas anoxopodis* pv *vignicola* 2625 (c: control, d: tested compound).

Poisoned food technique

The fungal species selected for these *in vitro* assays were: *A. alternata* RF8, *B. oryzae*, *B. cinerea* F411, *C. beticola*, *C. dematium* fsp *circinans* X, *F. oxysporum* fsp *lactucae* L55, *F. roseum*, *P. graminea*, *P. oryzae* BA43, *R. solani* and *S. sclerotiorum*.

The sensitivity of the tested fungi to *Perilla frutescens* compounds was evaluated as growth inhibition using concentrations of 100, 1000 and 2000 µg mL^{-1} for the PA and PK type extracts; of 200 and 500 µg mL^{-1} for the PA and PK type essential oils and 100 µg mL^{-1} for commercial PA. Unfortunately, synthetic PK was not tested because of the difficulties to obtain a sufficient amount to perform this kind of assys.

The effect of the natural compounds were compared with that of three fungicides: azoxystrobin (1 µg mL^{-1}) against *P. teres* and *P. oryzae*; cyprodinil (1 µg mL^{-1}) towards *B. cinerea* F411 and *A. alternata* RF8; and prochloraz (0.1 µg mL^{-1}) for the other fungi.

As reported in Tables 4.1-4.4 all samples exhibited a broad spectrum of activity against the analyzed fungi.

The activity data for the crude extracts obtained from the two perilla varieties are given in Tables 4.1-4.2. Both extracts at the lower concentration (100 µg mL^{-1}) did not show any relevant activity against the tested fungi.

The best results were observed after the 3^{th} day of incubation towards *C. beticola* with inhibition of 30.1% and 35.2% respectively, and for *A. alternata* RF8 with inhibition values of 21.4% and 20.2%.

In the case of the last pathogen, PA-Ex at 1000 µg mL^{-1} exhibited 100% inhibition at 3 days after incubation, the same effectiveness was also shown on *S. sclerotiorum*.

PK-Ex at the same concentration, completely inhibited the growth of *C. beticola* after 3 days of incubation, and of *B. oryzae*, *P. graminea* and *S. sclerotiorum* also after 5 days. It was found that PA-Ex and PK-Ex at 2000 µg mL^{-1} exhibited potent inhibitory activity (100%) on *A. alternata* RF8 5 and 3 days after incubation, respectively. A complete inhibition of *C. beticola* until the 7^{th} day was also observed in presence of both the extracts at this concentration.

PA-Ex at 2000 µg mL^{-1} exhibited 100% antifungal effect against *P. graminea* and *P. oryzae* BA43 (3^{th} day), these fungi were also completely stopped by PK-Ex 2000 µg mL^{-1} until 5 and 7 days of observation, respectively. *B. cinerea* F411 was totally inhibited at 3 days by PA-Ex at 2000 µg mL^{-1} while *B. oryzae* BA43 by PK-Ex at the same concentration until the 5^{th} day.

The less sensitive fungi were found to be *C. dematium* fsp *circinans* X, *R. solani* and *F. oxysporum* fsp *lactucae* L55, in fact the most active compound (PA-EX at 2000 µg. mL^{-1}), induced a moderate inhibition by 47.6%, 50% and 63.6% at 3 days of observation.

S. sclerotiorum was found to be sensible to DMSO at the highest concentration.

In the following figure 4.3a and 4.3b the sensitivity of some pathogens towards the tested crude extracts is graphically reported.

Mycelial discs totally inhibited by the compounds were transferred on not amended medium to evaluate the actual effect of the treatment. That was considered fungicidal if the fungi were unable to growth and fungistatic if the growth rastarted.

It was found that both the extracts at 2000 µg mL^{-1} exhibited fungistatic activity on *C. beticola*, moreover PK-Ex was also fungistatic against *P. graminea* and *P. oryzae* BA43.

Commercial PA showed complete mycelial inhibition was observed in the presence of against *A. alternata* RF8, *S. sclerotiorum* and *C. beticola* at 3, 5 and 7days after incubation, respectively. It showed also high antifungal activity at 3 days on *P. graminea* (100%), *B. oryzae* (95.8%), *R. solani* (88.9%) and *F. oxysporum* fsp *lactucae* L55 (74.7%). At the same period of observation, moderate effectiveness was exhibited against *B. cinerea* F411, *C. dematium* fsp *circinans* X and *P. oryzae* BA43, with percentage of mycelial growth inhibition of 65.7%, 53.7% and 47.1%, respectively.

Table 4.1 Percent growth inhibition on phytopathogenic fungi induced by PA and PK type extracts at different concentrations after 3, 5 and 7 days of incubation at 24±2°C.

Fungal species	Treatment	Concentration (µg mL^{-1})	Days		
			3	5	7
Alternaria alternata RF8	PA-Ex	100	21.4±2.1^{c}	12.9±4.5^{d}	8.5±2.0^{c}
		1000	100.0±0.0^{a}	59.5±1.9^{c}	45.6±2.1^{b}
		2000	100.0±0.0^{a}	100.0±0.0^{a}	84.1±3.4^{a}
	PK-Ex	100	20.2±3.1^{c}	6.4±3.4^{d}	4.0±2.1^{c}
		1000	63.9±3.4^{b}	43.3±4.1^{c}	29.8±0.5^{b}
		2000	100.0±0.0^{a}	85.5±3.2^{ab}	71.5±2.4^{a}
	PA	100	100.0±0.0^{a}	66.6±1.5^{c}	49.1±0.4^{b}
Cercospora beticola	PA-Ex	100	30.1±3.8^{b}	18.0±2.5^{b}	18.5±6.0^{d}
		1000	83.3±16.7^{a}	87.5±12.5^{a}	67.0±11.3^{c}
		2000	100.0±0.0^{a}	100.0±0.0^{a}	100.0±0.0^{a}
	PK-Ex	100	35.2±10.2^{b}	26.5±4.2^{b}	19.7±1.6^{d}
		1000	100.0±0.0^{a}	94.4±5.6^{a}	74.9±1.4^{ac}
		2000	100.0±0.0^{a}	100.0±0.0^{a}	100.0±0.0^{a}
	PA	100	100.0±0.0^{a}	100.0±0.0^{a}	100.0±0.0^{ab}
Colletotrichum dematium fsp *circinans* X	PA-Ex	100	11.1±11.1^{cd}	8.9±8.9^{c}	6.5±6.5^{bc}
		1000	19.0±3.2^{c}	0.0±0.0^{c}	0.0±0.0^{d}
		2000	47.6±2.4^{ab}	3.2±4.8^{a}	12.5±4.9^{a}
	PK-Ex	100	0.0±0.0^{d}	0.0±0.0^{c}	0.0±0.0^{d}
		1000	18.5±18.5^{c}	9.2±9.2^{c}	0.0±0.0^{d}
		2000	35.7±11.7^{b}	20.4±15.2^{b}	7.5±7.5^{ab}
	PA	100	53.7±2.1^{a}	0,0±0,0^{c}	1.3±1.3^{cd}
Pyricularia oryzae BA43	PA-Ex	100	8.8±4.6^{d}	5.2±2.3^{c}	5.1±2.6^{c}
		1000	22.5±1.3^{cd}	20.7±0.7^{c}	19.8±1.0^{c}
		2000	100.0±0.0^{a}	67.0±0.4^{b}	52.5±0.9^{b}
	PK-Ex	100	7.5±4.3^{d}	10.6±2.4^{c}	3.1±3.1^{c}
		1000	71.0±0.5^{b}	63.6±1.8^{b}	53.1±0.0^{b}
		2000	100.0±0.0^{a}	100.0±0.0^{a}	100.0±0.0^{a}
	PA	100	47.1±0.8^{bc}	30.3±3.0^{c}	25.2±0.9^{C}
Rhizoctonia solani	PA-Ex	100	0.9±0.9^{c}	1.8±0.9^{d}	0.0±0.0^{c}
		1000	37.5±2.2^{b}	38.0±2.4^{b}	26.2±3.3^{b}
		2000	50.0±0.0^{b}	26.8±1.8^{bc}	24.4±4.7^{b}
	PK-Ex	100	0.0±0.0^{c}	3.7±0.9^{d}	0.0±0.0^{c}
		1000	7.5±4.3^{c}	13.6±2.3^{cd}	0.0±0.0^{c}
		2000	5.6±5.6^{c}	5.6±5.6^{d}	2.6±2.6^{c}
	PA	100	88.9±0.0^{a}	71.7±0.7^{a}	60.1±1.4^{a}

Results are mean ± SE values for three replicates. Means in the same column by same letter are not significantly different according to Duncan's multiple range tests ($p<0.05$).

Table 4.2 Percent growth inhibition on phytopathogenic fungi induced by PA and PK type extracts and commercial PA, at different concentrations, after 3 and 5 days of incubation at 24±2°C.

Fungal species	Treatment	Concentration (µg mL^{-1})	Days	
			3	5
Bipolaris oryzae	PA-Ex	100	6.4±1.8^{d}	6.8±3.4^{c}
		1000	41.4±0.7^{bc}	26.2±1.3^{bc}
		2000	59.7±5.0^{b}	41.5±1.6^{b}
	PK-Ex	100	19.1±3.0^{cd}	10.2±0.0^{c}
		1000	100.0±0.0^{a}	100.0±0.0^{a}
		2000	100.0±0.0^{a}	100.0±0.0^{a}
	PA	100	95.8±4.2^{a}	81.6±6.2^{a}
Botrytis cinerea F411	PA-Ex	100	0.0±0.0^{d}	0.0±0.0^{c}
		1000	35.6±2.2^{c}	8.4±4.2^{bc}
		2000	100.0±0.0^{a}	41.4±8.1^{a}
	PK-Ex	100	1.5±1.5^{d}	7.7±7.7^{c}
		1000	24.0±4.5^{cd}	7.7±7.7^{bc}
		2000	60.4±5.5^{b}	22.3±9.6^{b}
	PA	100	65.7±0.5^{b}	53.0±0.8^{a}
Fusarium oxysporum fsp *lactucae* L55	PA-Ex	100	5.8±0.0^{d}	0.0±0.0^{d}
		1000	30.7±3.7^{bc}	17.8±2.5^{bc}
		2000	63.6±0.0^{a}	48.7±0.7^{a}
	PK-Ex	100	10.4±1.9^{d}	0.0±0.0^{d}
		1000	13.9±0.2^{cd}	5.1±0.6^{cd}
		2000	33.3±3.0^{b}	26.8±3.2^{b}
	PA	100	74.7±0.3^{a}	58.5±0.7^{a}
Pyrenophora graminea	PA-Ex	100	1.2±1.2^{c}	0.0±0.0^{b}
		1000	51.4±1.4^{b}	33.3±2.1^{b}
		2000	100.0±0.0^{a}	76.0±0.5^{a}
	PK-Ex	100	0.0±0.0^{c}	0.0±0.0^{b}
		1000	100.0±0.0^{a}	100.0±0.0^{a}
		2000	100.0±0.0^{a}	100.0±0.0^{a}
	PA	100	100.0±0.0^{a}	81.6±5.1^{a}
Sclerotinia sclerotiorum	PA-Ex	100	13.9±5.2^{b}	0.0±0.0^{c}
		1000	100.0±0.0^{a}	55.9±2.9^{b}
		2000	0.0±0.0^{b}	0.0±0.0^{c}
	PK-Ex	100	0.0±0.0^{b}	0.0±0.0^{c}
		1000	100.0±0.0^{a}	100.0±0.0^{a}
		2000	0.0±0.0^{b}	0.0±0.0^{c}
	PA	100	100.0±0.0^{a}	100.0±0.0^{a}

Results are mean ± SE values for three replicates. Means in the same column by same letter ar not significantly different according to Duncan's multiple range tests ($p<0.05$).

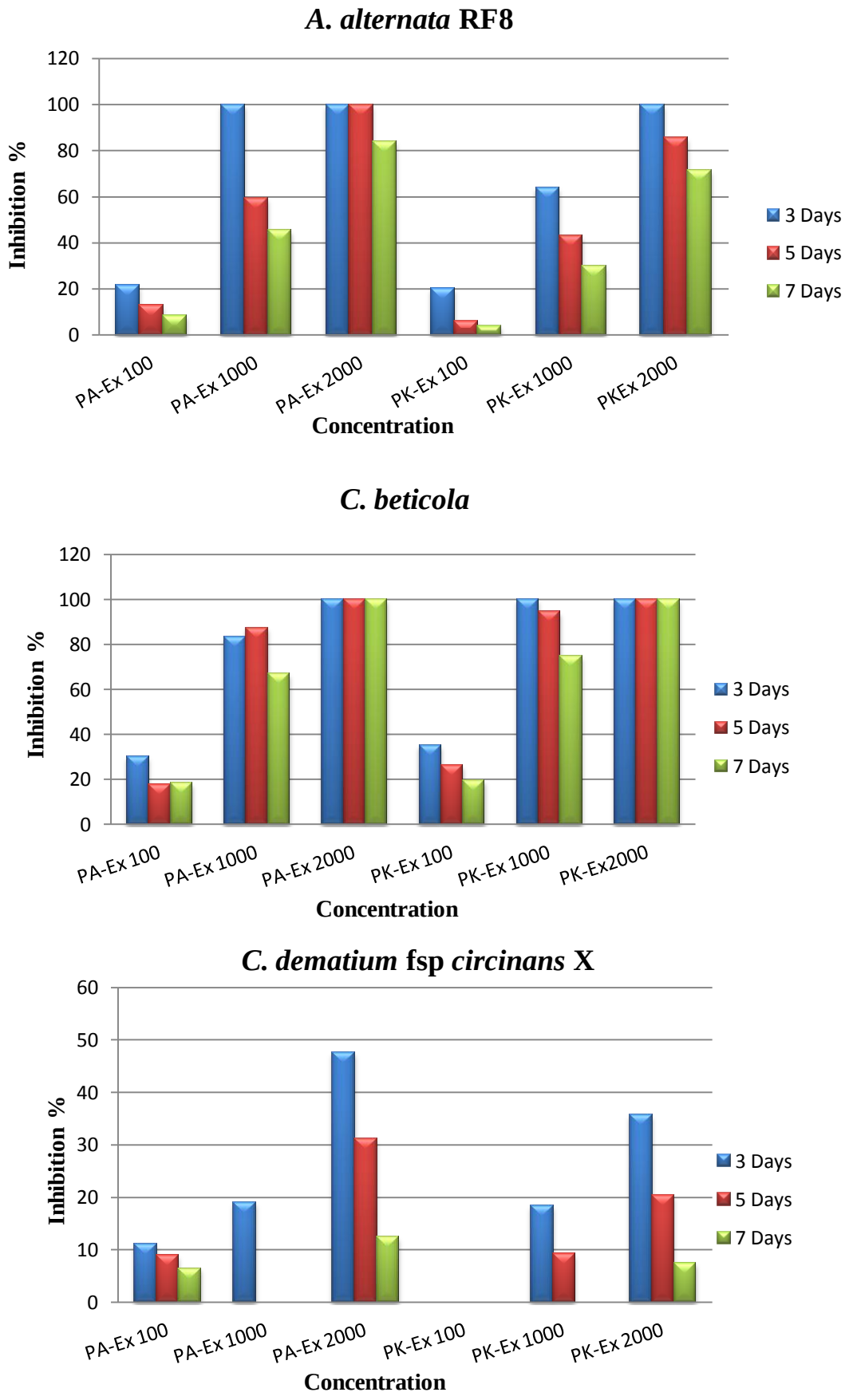


Figure 4.3a Growth inhibition induced by crude extracts PA and PK type on *A. alternata* RF8, *C. beticola* and *C. dematium* fsp *circinans* X. Concentration expressed as µg mL^{-1}.

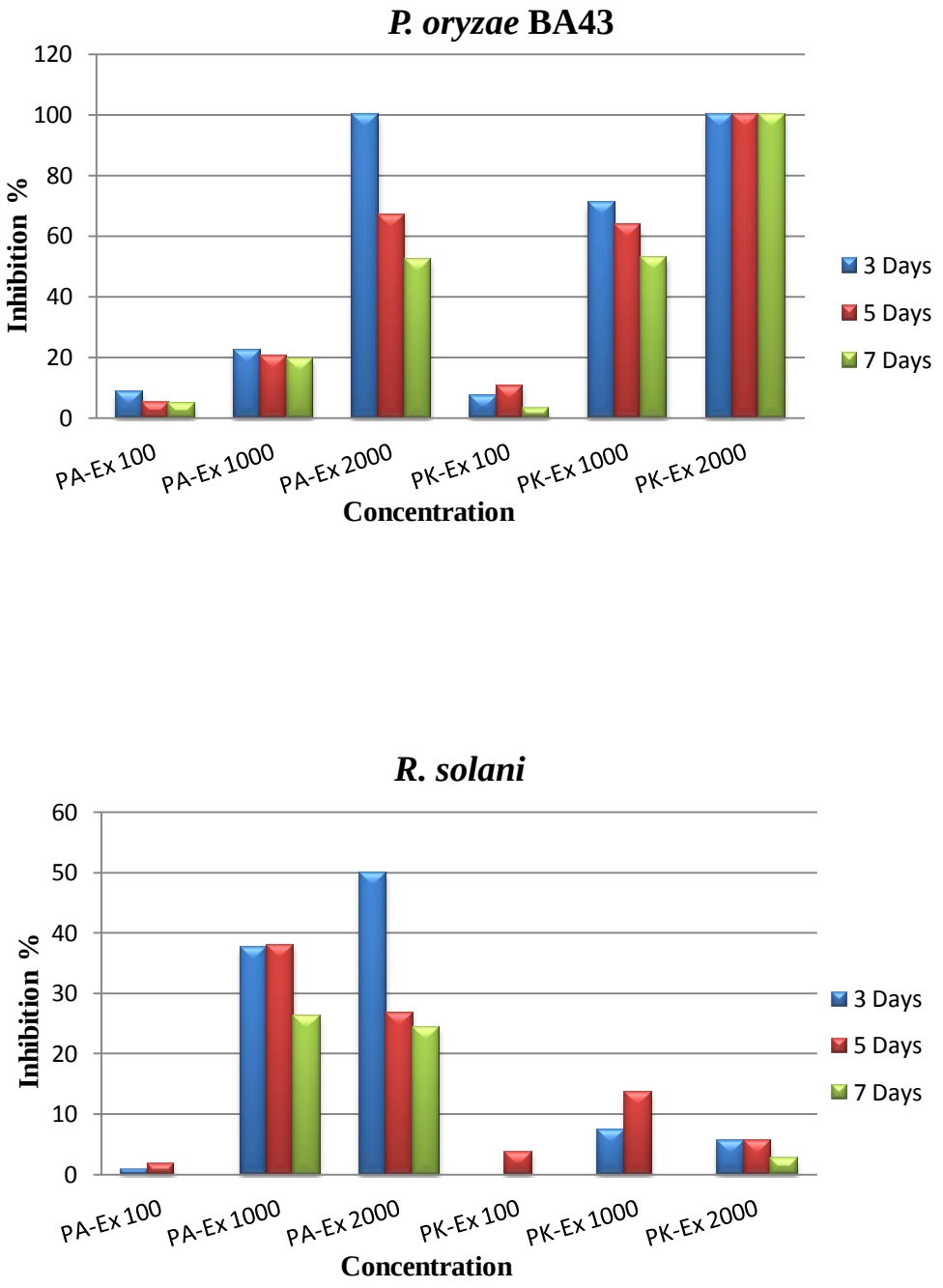


Figure 4.3b Growth inhibition induced by crude extracts PA and PK type on *P. oryzae* BA43 and *R. solani.*
Concentration expressed as µg mL^{-1}.

The antifungal activity of the essential oils obtained from the two perilla chemotypes are reported in Tables 4.3-4.4.

PA and PK type essential oils induced a mild to high antifungal efficacy against the tested plant pathogens.

After 3 days of incubation, both oils at lower concentration inhibited completely *A. alternata* RF8, while *C. beticola* was inhibited by PA-EO, only. 5 days after incubation, both oils exhibited 100% antifungal effect against *S.sclerotiorum*. At the same observation time and concentration, *P. teres* was inhibited by PA-EO while *P. graminea* and *P. oryzae* BA43 by PK-EO. At the 7th day of incubation *P. teres* was totally stopped by PK-EO, only.

The most resistent species to PK-EO at 200 µg mL^{-1} at 3 days after incubation were: *F. roseum* (23%), *C. dematium* fsp *circinans* X (46.1%), *C. beticola* (50%), *F. oxysporum* fsp *lactucae* L55 (66.1%) and *R. solani* (68.4%).

5 days after incubation, *B. cinerea* F411, *B. oryzae, P. graminea* and *S. sclerotiorum* were found to be the most inhibited pathogens by both essential oils at 500 µg mL^{-1}. A completely inhibition of *F. oxysporum* fsp *lactucae* L55 and *F. roseum* was observed in the presence of PA-EO, only.

The tested essential oils at highest concentration demonstrated complete inhibition at the 7th day of incubation on *A. alternata* RF8, *C. beticola, P. oryzae* BA43 and *P. teres*.

A 100% inhibition of *R. solani* until the 7th day was also observed for PA-EO.

In the Figure 4.4-4.7, inhibition growth of some phytopathogen fungi induced by PA and PK type essential oils is reported.

The less sensitive species to PK-EO at 500 µg mL^{-1} were found to be *C. dematium* fsp *circinans* X (70%) and *F. roseum* (80.3%), at 5 days after incubation.

In addition, it was found that PA-EO at the highest concentration exerted a fungicidal effect on *A. alternata* RF8, *B. oryzae, C. beticola*, *P. graminea*, *P. oryzae* BA43 and *P. teres*. On the contrary at the same concentration, PK-EO exhibited a fungistatic activity against the six last mentioned fungi and *S. sclerotiorum*.

Table 4.3 Percent growth inhibition on phytopathogenic fungi induced by PA and PK type essential oils at different concentrations after 3, 5 and 7 days of incubation at 24±2°C.

Fungal species	Treatment	Concentration (µg mL^{-1})	Days		
			3	5	7
Alternaria alternata RF8	PA-EO	200	100.0±0.0^{a}	84.8±2.3^{b}	70.4±2.5^{b}
		500	100.0±0.0^{a}	100.0±0.0^{a}	100.0±0.0^{a}
	PK-EO	200	100.0±0.0^{a}	78.4±2.7^{b}	70.4±2.5^{b}
		500	100.0±0.0^{a}	100.0±0.0^{a}	100.0±0.0^{a}
	Cyprodinil	1	100.0±0.0^{a}	100.0±0.0^{a}	94.8±0.0^{a}
Cercospora beticola	PA-EO	200	100.0±0.0^{a}	76.0±2.6^{ab}	68.1±0.8^{b}
		500	100.0±0.0^{a}	100.0±0.0^{a}	100.0±0.0^{a}
	PK-EO	200	50.0±0.0^{b}	47.8±1.1^{c}	46.9±0.7^{b}
		500	100.0±0.0^{a}	100.0±0.0^{a}	100.0±0.0^{a}
	Prochloraz	0.1	57.9±4.0^{b}	54.4±3.1^{bc}	43.8±2.0^{b}
Colletotrichum dematium fsp *circinans* X	PA-EO	200	64.6±1.0^{b}	31.8±1.8^{c}	20.9±3.3^{bc}
		500	100.0±0.0^{a}	100.0±0.0^{a}	95.9±0.0^{a}
	PK-EO	200	46.1±2.1^{b}	19.4±2.1^{c}	10.0±1.7^{c}
		500	100.0±0.0^{a}	70.0±0.7^{ab}	46.8±2.5^{b}
	Prochloraz	0.1	55.6±5.6^{b}	46.7±1.1^{bc}	40.5±4.8^{bc}
Pyrenophora teres	PA-EO	200	100.0±0.0^{a}	100.0±0.0^{a}	91.8±1.2^{a}
		500	100.0±0.0^{a}	100.0±0.0^{a}	100.0±0.0^{a}
	PK-EO	200	100.0±0.0^{a}	100.0±0.0^{a}	100.0±0.0^{a}
		500	100.0±0.0^{a}	100.0±0.0^{a}	100.0±0.0^{a}
	Azoxystrobin	1	68.9±0.5^{b}	65.0±0.9^{b}	53.7±0.7^{b}
Pyricularia oryzae BA43	PA-EO	200	81.8±0.0^{b}	52.6±0.3^{b}	40.0±0.5^{b}
		500	100.0±0.0^{a}	100.0±0.0^{a}	100.0±0.0^{a}
	PK-EO	200	100.0±0.0^{a}	100.0±0.0^{a}	94.8±3.0^{a}
		500	100.0±0.0^{a}	100.0±0.0^{a}	100.0±0.0^{a}
	Azoxystrobin	1	100.0±0.0^{a}	85.3±3.0^{a}	79.1±1.5^{a}
Rhizoctonia solani	PA-EO	200	83.4±1.6^{ab}	71.5±1.8^{b}	51.0±3.1^{b}
		500	100.0±0.0^{a}	100.0±0.0^{a}	100.0±0.0^{a}
	PK-EO	200	68.4±2.8b^{c}	62.7±0.9^{b}	45.6±1.8^{b}
		500	100.0±0.0^{a}	100.0±0.0^{a}	98.2±1.8^{a}
	Prochloraz	0.1	55.8±3.2^{c}	57.1±0.4^{b}	47.6±0.7^{b}

Results are mean ± SE values for three replicates. Means in the same column by same superscript letter are not significantly different according to Duncan's multiple range tests ($p<0.05$).

Table 4.4 Percent growth inhibition on phyto-pathogenic fungi induced by PA and PK type essential oils at different concentrations after 3 and 5 days of incubation at 24±2°C.

Fungal species	Treatment	Concentration (µg mL^{-1})	Days	
			3	5
Bipolaris oryzae	PA-EO	200	93.9±0.1^{a}	71.1±0.3^{abc}
		500	100.0±0.0^{a}	100.0±0.0^{a}
	PK-EO	200	83.6±1.2^{a}	67.7±0.9^{ac}
		500	100.0±0.0^{a}	100.0±0.0^{ab}
	Prochloraz	0.1	37.9±1.7^{b}	23.8±1.4^{d}
Botrytis cinerea F411	PA-EO	200	96.7±3.3^{b}	66.3±1.8^{b}
		500	100.0±0.0^{a}	100.0±0.0^{a}
	PK-EO	200	98.4±1.6^{ab}	74.5±2.1^{b}
		500	100.0±0.0^{a}	100.0±0.0^{a}
	Cyprodinil	1	97.0±1.6^{b}	94.2±0.9^{a}
Fusarium oxysporum fsp *lactucae* L55	PA-EO	200	80.7±1.5^{bc}	63.9±1.8^{b}
		500	100.0±0.0^{a}	100.0±0.0^{a}
	PK-EO	200	66.1±1.8^{c}	57.8±1.8^{b}
		500	100.0±0,0^{a}	92.2±1.2^{a}
	Prochloraz	0.1	87.8±0.3^{ab}	84.4±0.7^{a}
Fusarium roseum	PA-EO	200	81.6±3.0^{a}	55.8±4.6^{b}
		500	100.0±0.0^{a}	100.0±0.0^{a}
	PK-EO	200	23.0±3.8^{b}	7.5±2.7^{c}
		500	85.1±1.6^{a}	80.3±1.4^{ab}
	Prochloraz	0.1	85.0±0.2^{a}	82.3±0.7^{ab}
Pyrenophora graminea	PA-EO	200	100.0±0.0^{a}	80.8±4.3^{a}
		500	100.0±0.0^{a}	100.0±0.0^{a}
	PK-EO	200	100,0±0,0^{a}	100,0±0.0^{a}
		500	100.0±0.0^{a}	100.0±0.0^{a}
	Prochloraz	0.1	35.6±1.1^{b}	20.3±0.9^{b}
Sclerotinia sclerotiorum	PA-EO	200	100.0±0.0^{a}	100.0±0.0^{a}
		500	100.0±0.0^{a}	100.0±0.0^{a}
	PK-EO	200	100.0±0.0^{a}	100.0±0.0^{a}
		500	100.0±0.0^{a}	100.0±0.0^{a}
	Prochloraz	0.1	100.0±0.0^{a}	100.0±0.0^{a}

Results are mean ± SE values for three replicates. Means in the same column by same letter are not significantly different according to Duncan's multiple range tests ($p<0.05$).

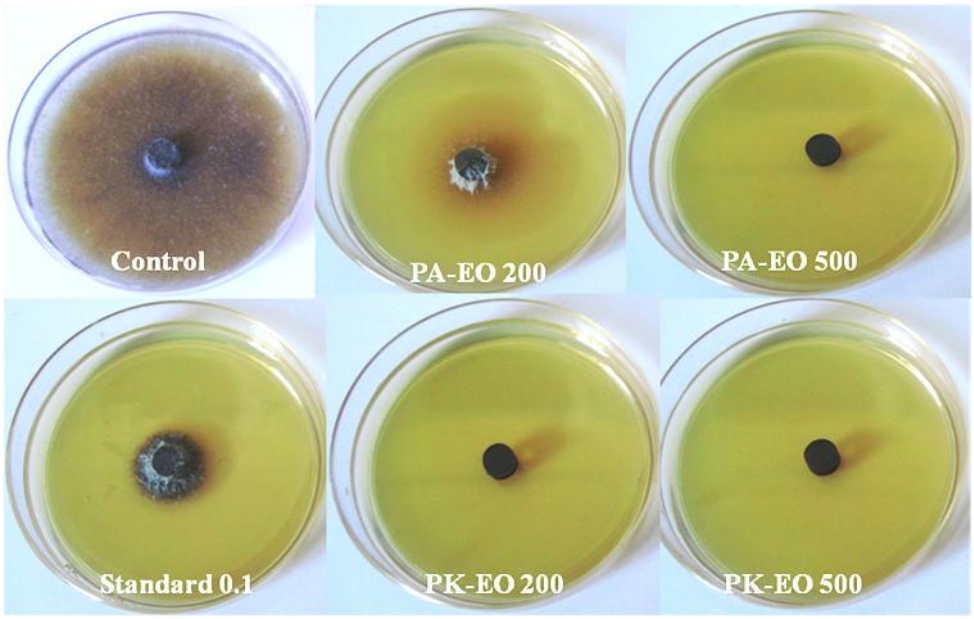


Figure 4.4 Inhibition of *P graminea* growth induced by PA and PK type essential oils, after 7 days of incubation. Standard compound: prochloraz (0.1 µg mL^{-1}).
Concentration expressed as µg mL^{-1}.

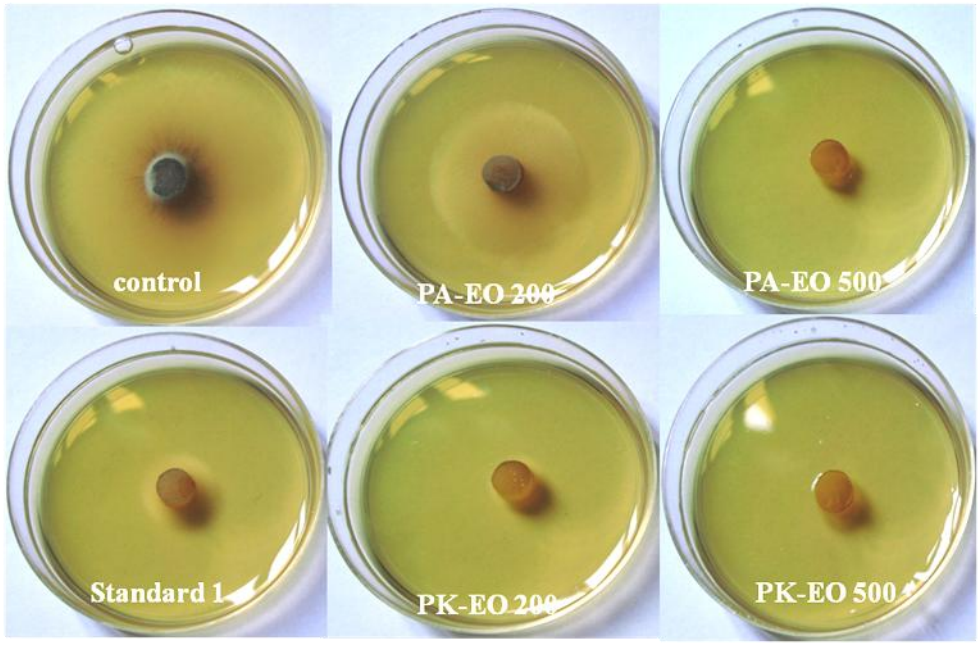


Figure 4.5 Inhibition of *P oryzae* BA43 growth induced by PA and PK type essential oils, after 7 days af incubation.Standard compound: azoxystrobin (1 µg mL^{-1}).
Concentration expressed as µg mL^{-1}.

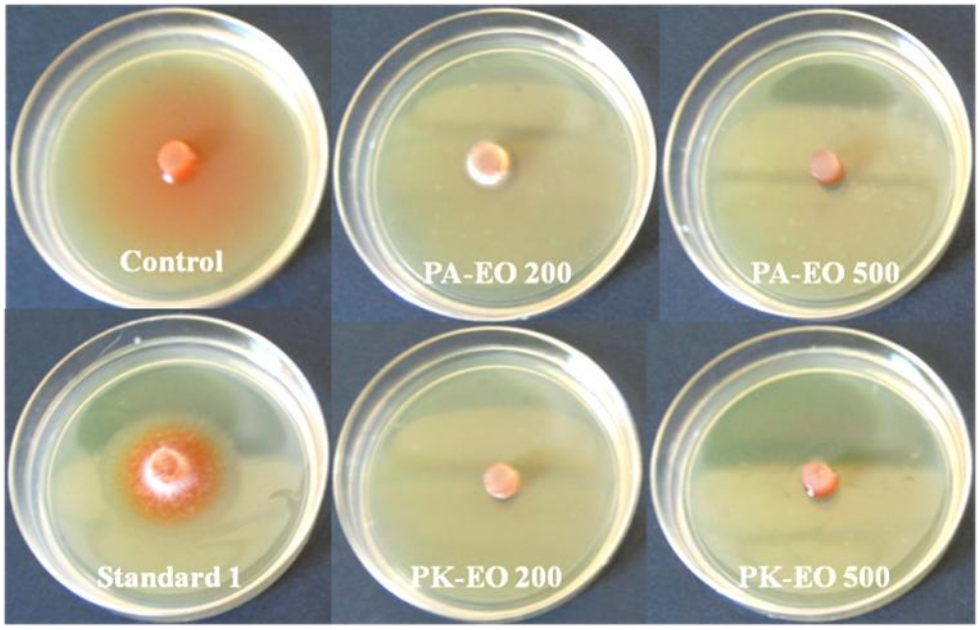


Figure 4.6 Inhibition of *P teres* growth induced by PA and PK type essential oils, after 7 days of incubation. Standard compound: azoxystrobin (1 µg mL^{-1}).
Concentration expressed as µg mL^{-1}.

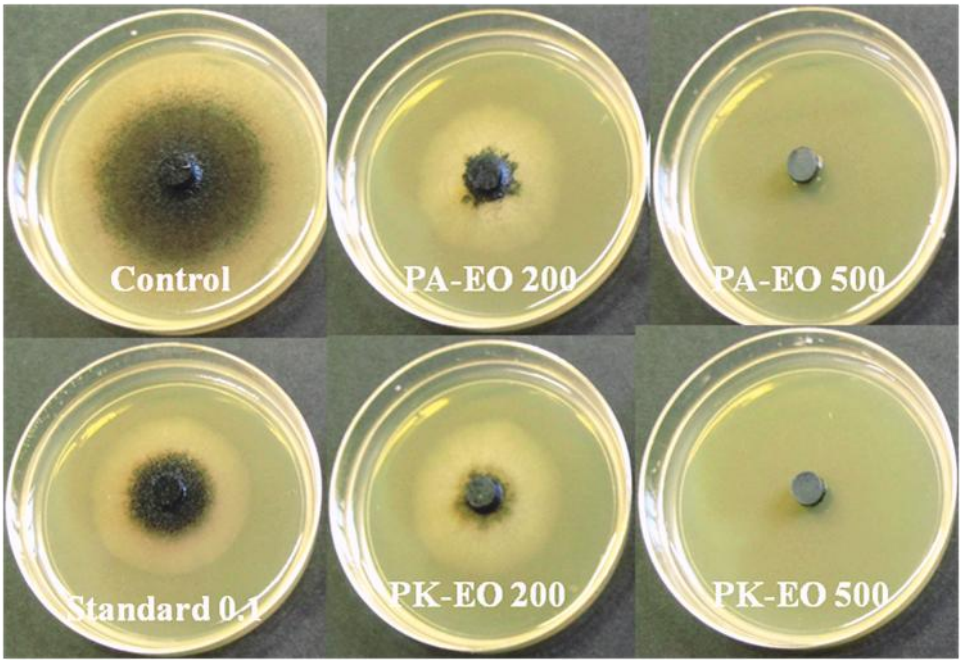


Figure 4.7 Inhibition growth of *R. solani* induced by PA and PK type essential oils, after 7 days of incubation. Standard compound: prochloraz (0.1 µg mL^{-1}).
Concentration expressed as µg mL^{-1}.

4.3.2 *In vivo* preventive activity

To examine *in vivo* the potential preventive activity of the crude extracts and essential oils PA and PK-type and pure PA, *Sphaerotheca fuliginea*/*Cucumis sativus* (powdery mildew) was chosen among some of the most economically relevant and widespread pathogen-host plant combination. Powdery mildew makes a white powdery growth on the upper surfaces of leaves and on the stems of the plants. Infected areas are often stunted and distorted, and may drop prematurely from the plant. Antifungal efficacy was assessed by the presence or absence of disease area on the tested plants (Figure 4.8), and compared with untreated control. No phytotoxic effects were observed on the cucumber plants by any of the treatments applied.

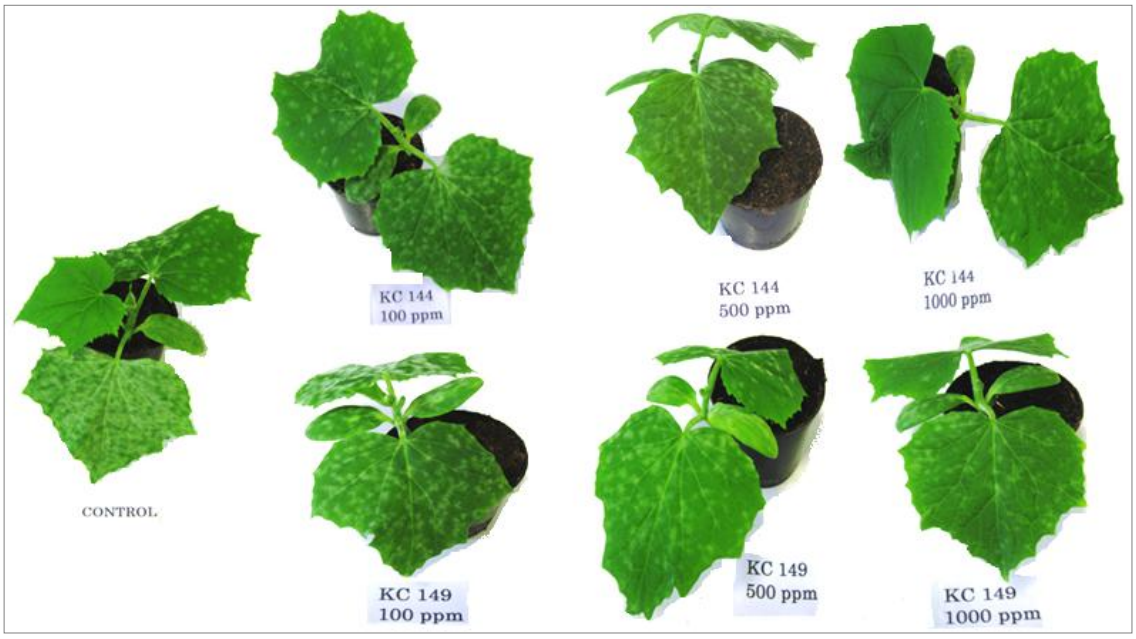


Figure 4.8 *In vivo* preventive activity of PA and PK type essential oils (KC144 and KC149, respectively) at different concentrations against *Sphaerotheca fuliginea* on cucumber plants, at 8 days after the treatment.

The activities of the compounds and the commercial fungicide metrafenone, were expressed as percent inhibition of infection and are reporteted in Figure 4.9. Few disease symptoms were observed on the plants when the reference standard was administered at the concentration of 0.01 μg mL^{-1}. Instead, the Perilla derived compounds revaled a wide

range of antifungal activity at 8 and 10 days after the treatment.

The best control was determined for all samples 8 days post-treatment. Disease severity was significantly reduced by pure PA at concentration of 100 µg mL^{-1} to 25%, compared with 84% on non-treated plants. The efficacy of commercial PA markedly increased in a concentration-dependent manner. PA and PK type extracts at 1000 µg mL^{-1} showed good activity in controlling the powdery mildew, both the compounds reduced disease symptoms respectively by 61.9% and 57.8%. On the contrary, the extracts were only moderate active at lowest tested concentration (36.8% and 32.7%).

In the plants treated with a highest concentration (1000 µg mL^{-1}), the essential oils PA and PK type reduced the pathogen development of 42% and 56%. Their activity resulted scanty at lowest concentrations 10 days after the treatment, in fact the disease was inhibited of 28% and 22%, only.

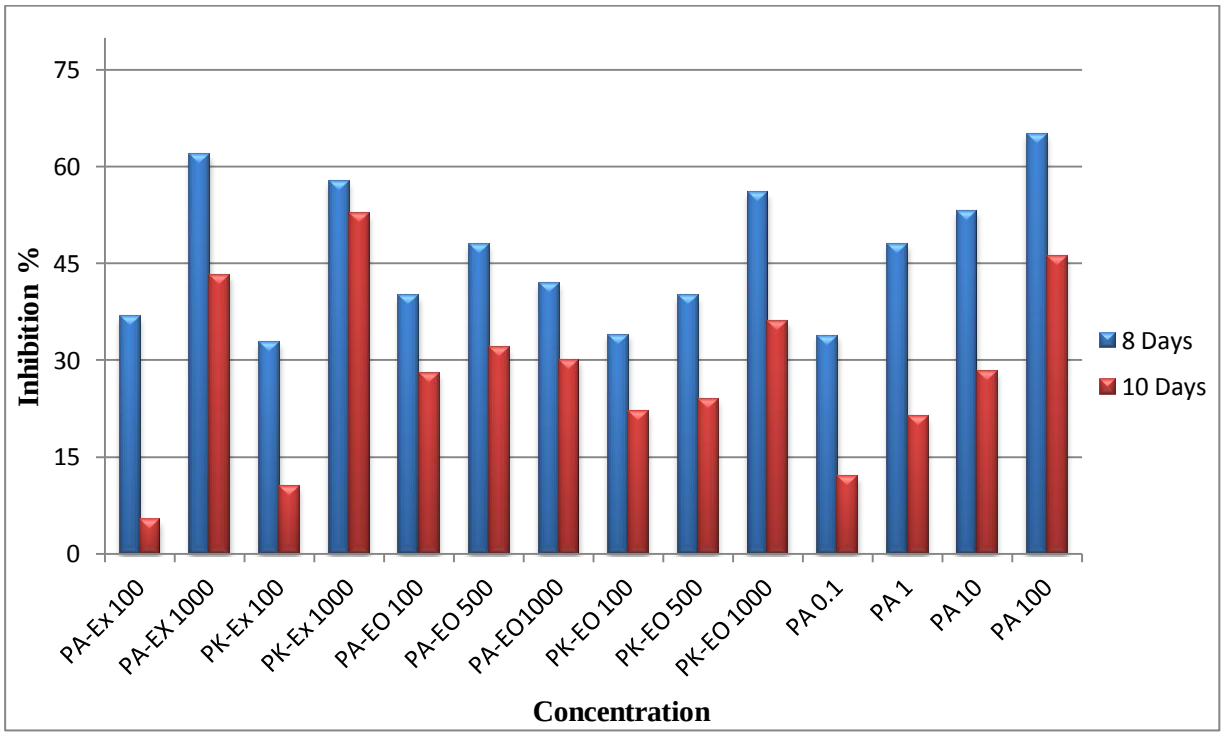


Figure 4.9 Preventive activity of crude extracts (PA-Ex and PK-Ex), essential oils (PA-EO and PK-EO) and commercial PA at different concentrations against *Sphaerotheca fuliginea* on *Cucumis sativus*, 8 and 10 days after the treatment. Concentration expressed as µg mL^{-1}.

4.3.3 Antifungal activity against *Cladosporium* spp

Among the analyzed fungi particular attention was given to some strains belonging to the genus *Cladosporium*.

Cladosporium spp, which comprises more than 772 names, has been studied extensively in recent years. *C. cladosporioides* is a very common, cosmopolitan, saprobic species, which causes pod rot and blight of pea. It often occurs as a secondary invader on necrotic parts of many different host plants, has been isolated from air, soil, textiles and several other substrates, and is a common endophytic or quiescent fungus. In the past *C. cladosporioides* has been reported to be involved in several pulmonary and cutaneous infections and other human health problems (Bensch *et al*., 2010).

C. cucumerinum has been described to be the causal agent of scab, important disease of cucumber (*Cucumis sativus* L.) worldwide. Scab can cause serious losses for cucumber production, especially in protected culture such as high tunnel production (Zhang *et al*., 2010).

Following the sensitivity tested strains to the single treatments is reported.

Disk diffusion method

At first the antifungal property of PA-EO, PK-Ex and pure PK was evaluated *in vitro* by paper disk diffusion assays, against *C. cladosporioides* IPV-F167, at concentrations of 250, 500, 1000, 2000 µg mL^{-1}. Antifungal activity was assessed by measuring the diameter of the inhibition zone around the disks (in millimeters) for the test organism comparing to the control (Figure 4.10).

In these experiments, assayed compounds at lower concentrations did not show any relevant activity against the tested fungus. The highest activity was observed for all samples at 2000 µg mL^{-1}. At 7 days after incubation, the values of inhibition zones were found to be: 9.0, 10.0 and 11.0 mm for PA-EO, PK-Ex and PK, respectively.

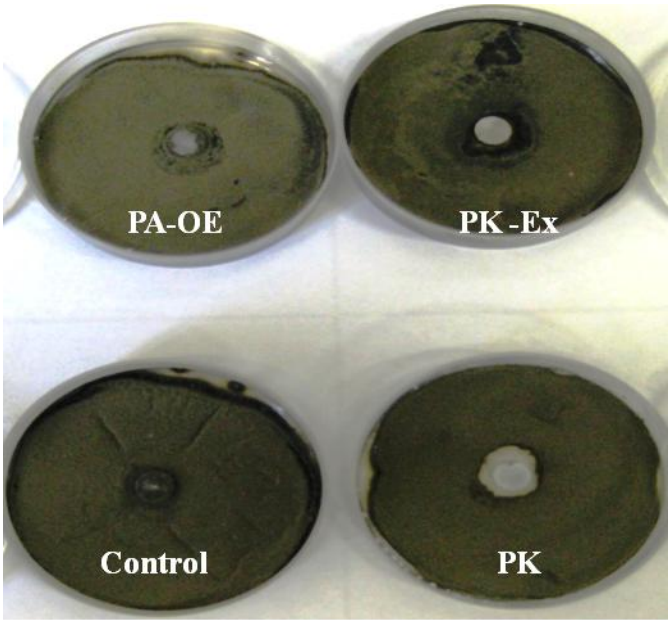


Figure 4.10 Inhibition zones caused by tested compounds (2000 µg mL-1) on *C. cladosporioides* IPV-F167.

Bioautography on thin-layer plates

In the direct bioautography on TLC plates, *C. cladosporioides* IPV-F167, due to good sporulation was chosen to determine the antifungal activity of crude extracts (PA-Ex and PK-Ex), essential oils (PA-EO and PK-EO), synthetic PK and perillaldehyde.

The efficacy was expressed as minimum amount required for the inhibition of fungal growth and are reported in Table 4.5. Prochloraz, a commercial antifungal agent, was also tested as positive control.

Table 4.5 Antifungal activity of Perilla compounds against *C. cladosporioides* IPV-F167 on TLC plates after 5 days of incubation at 24±2°C.

Compound	**Antifungal activity (µg/spot)**[a]
	C. cladosporioides IPV-F167
PA-Ex	10
PK-Ex	*b*
PA-EO	10
PK-EO	1
PA	50
PK	50
Prochloraz (positive control)	10^{-1}

[a]Minimum amount required for the inhibition of fungal growth on thin-layer chromatography plates.
[b] Inactive at 100 µg

As can be seen in Figure 4.11 the inhibition of fungal growth appeared as clear zones against a dark background. In these experiments both oils exhibited antifungal activity at 1µg (PK-EO) and 10 µg (PA-EO). Among the extracts, PA-Ex was active at 10 µg while PK-Ex displayed no detectable activity. The minimum amount required to show an inhibition zone for commercial PA and pure PK were 50 µg, while the synthetic fungicide prochloraz was active at 0.1 µg.

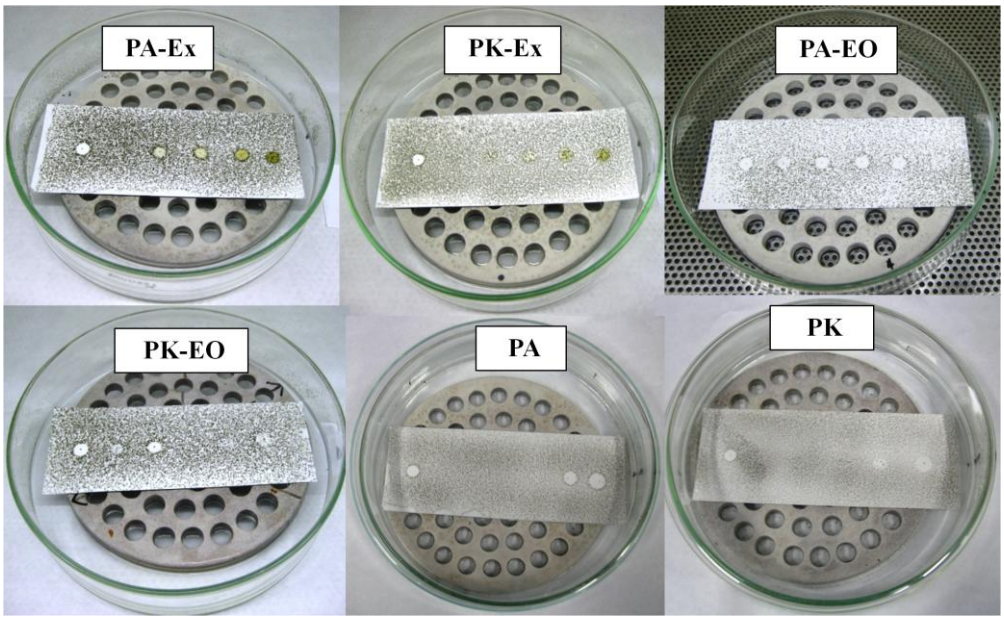


Figure 4.11 *Bioautography* assays on *C. cladosporioides* IPV-F167 with tested compounds.

Spore germination assay

Both essential oils and commercial PA were also investigated for their ability to inhibite germination of *C. cladosporioides* IPV-F167 conidia. The results expressed in terms of percent inhibition of spore germination, are shown in Table 4.6.

The observation by light microscope of fungal spores on agar media amended with DMSO at the highest concentration, showed that the solvent did not inhibit the conidia germination. On the contrary, It was found a significant inhibition of spore germination by different concentrations of the oils. A 94.9% inhibition was observed at the highest concentration (500 µg mL^{-1}) of PA-EO. PK-EO also exhibited a potent inhibitory effect (75.1 and 86.7 % at 250 and 500 µg mL^{-1}, respectively).

On the other hand the essential oil PK type was not so efficient in inhibiting spore germination at lowest concentration, in fact the inhibition value was 48.6%.
Commercial PA at the tested concentration, instead, provided high effective inhibition by 79.6%.

Table 4.6 Inhibitory effect of different concentrations of PA and PK type essential oils and pure PA on *C. cladosporioides* IPV-F167 spores germination, after 24 hrs of incubation at 20±2°C .

Compound (µg mL^{-1})	**Inhibition of spores germination (%)**		
	100	**250**	**500**
Control	0	0	0
DMSO	-	-	0
PK-EO	48.6	75.1	86.7
PA-EO	74.5	86.5	94.9
PA	79.6	-	-

-, not tested.

Poisoned food technique

To evaluate the concentration-activity relationship of *Perilla frutescens* derived compounds and commercial PA, *C. cladosporioides* IPV-F167, *C. cladosporioides* CBS 574.78 A and *C. cucumerinum* were assayed by the poisoned food technique:
The antifungal activity of samples, expressed in terms of percentage of mycelial growth inhibition, was compared with that of prochloraz. Activity recorded after 3, 5 and 7 days of incubation at 24±2°C, is reported in Tables 4.7 and 4.8.
All compounds showed a broad spectrum of activity during the whole observation period.

Table 4.7 Percent inhibition of *Cladosporium* spp growth induced by PA and PK type extracts at different concentrations after 3, 5 and 7 days of incubation at 24±2°C.

Treatment	Concentration (µg mL^{-1})	*C. cladosporioides* IPV-F167			*C. cladosporioides* CBS 574,78 A			*C. cucumerinum*		
		3	5	7	3	5	7	3	5	7
PA- Ex	100	14.9±2.5^{de}	11.1±11.1^{e}	2.4±2.4^{f}	0.0±0.0^{d}	0.0±0.0^{c}	0.0±0.0^{d}	13.7±0.6^{c}	10.9±5.5^{d}	8.2±4.2^{d}
	1000	50.7±3.6^{b}	42.2±2.2^{cd}	37.8±0.8^{de}	40.7±3.7^{c}	18.8±1.2^{c}	29.0±7.0^{bc}	66.0±3.3^{b}	76.8±11.6^{b}	61.8±5.4^{c}
	2000	49.7±7.1^{b}	59.9±5.1^{b}	65.2±2.6^{ab}	100.0±0.0^{a}	76.6±0.3^{a}	69.49±4.2^{a}	100.0±0.0^{a}	79.6±4.6^{b}	71.7±8.8^{abc}
PK- Ex	100	0.0±0.0^{e}	3.3±3.3^{e}	0.0±0.0^{f}	0.0±0.0^{d}	1.5±7.6^{c}	0.5±0.0^{d}	0.0±0.0^{c}	3.3±3.3^{d}	1.4±1.4^{d}
	1000	37.3±2.3^{bc}	33.3±3.8^{d}	29.7±1.8^{e}	33.3±0.0^{c}	17.6±13.8^{c}	25.0±1.0^{c}	56.0±7.8^{b}	57.7±2.8^{c}	61.0±9.4^{c}
	2000	24.8±10.8^{cd}	50.8±8.9^{bc}	57.0±4.1^{bc}	71.43±0.0^{b}	69.8±0.4^{a}	67.9±3.9^{a}	68.0±8.4^{b}	57.5±3.8^{c}	64.6±1.0^{bc}
PA	100	100.0±0.0^{a}	88.4±1.0^{a}	74.6±1.9^{a}	100.0±0.0^{a}	81.7±9.4^{a}	62.4±2.4^{a}	100.0±0.0^{a}	100.0±0.0^{a}	86.1±2.4^{a}
Prochloraz	0.1	37.8±2.2^{bc}	43.5±4.1^{cd}	46.6±1.6^{cd}	47.0±1.5^{b}	46.4±2.1^{b}	43.5±1.6^{b}	100.0±0.0^{a}	83.6±2.1^{ab}	80.8±1.6^{ab}

Results are mean ± SE values for three replicates. Means in the same column by same letter are not significantly different according to Duncan's multiple range tests ($p<0.05$).

Table 4.8 Percent inhibition of *Cladosporium* spp growth induced by PA and PK type essential oils (EO) at different concentrations after 3, 5 and 7 days of incubation at 24±2°C.

Treatment	Concentration (µg mL^{-1})	*C. cladosporioides* IPV-F167			*C. cladosporioides* CBS 574,78 A			*C. cucumerinum*		
		3	5	7	3	5	7	3	5	7
PA-EO	100	79.6±1.9^{b}	53.1±1.6^{c}	47.0±1.5^{c}	40.3±2.1^{b}	30.7±1.3^{c}	23.9±1.8^{c}	65.6±2.9^{b}	72.7±0.0^{b}	57.0±4.3^{c}
	200	100.0±0.0^{a}	85.0±4.4^{b}	78.2±1.1^{b}	100.0±0.0^{a}	71.9±3.5^{b}	60.1±3.3^{b}	100.0±0.0^{a}	100.0±0.0^{a}	91.7±5.5^{b}
	300	100.0±0.0^{a}	100.0±0.0^{a}	100.0±0.0^{a}	100.0±0.0^{a}	100.0±0.0^{a}	100.0±0.0^{a}	100.0±0.0^{a}	100.0±0.0^{a}	100.0±0.0^{a}
	400	100.0±0.0^{a}	100.0±0.0^{a}	100.0±0.0^{a}	100.0±0.0^{a}	100.0±0.0^{a}	100.0±0.0^{a}	100.0±0.0^{a}	100.0±0.0^{a}	100.0±0.0^{a}
	500	100.0±0.0^{a}	100.0±0.0^{a}	100.0±0.0^{a}	100.0±0.0^{a}	100.0±0.0^{a}	100.0±0.0^{a}	100.0±0.0^{a}	100.0±0.0^{a}	100.0±0.0^{a}
PK-EO	100	62.2±2.3^{c}	50.0±0.9^{c}	48.5±6.6^{c}	49.5±6.0^{b}	36.6±2.4^{c}	26.6±3.4^{c}	67.3±3.7^{b}	72.7±2.6^{b}	63.1±6.6^{c}
	200	100.0±0.0^{a}	80.8±0.4^{b}	72.4±0.8^{b}	100.0±0.0^{a}	82.5±3.5^{b}	72.6±7.7^{b}	100.0±0.0^{a}	100.0±0.0^{a}	93.9±0.1^{ab}
	300	100.0±0.0^{a}	98.0±2.0^{a}	92.1±1.4^{a}	100.0±0.0^{a}	100.0±0.0^{a}	100.0±0.0^{a}	100.0±0.0^{a}	100.0±0.0^{a}	100.0±0.0^{a}
	400	100.0±0.0^{a}	97.9±2.1^{a}	95.5±4.5^{a}	100.0±0.0^{a}	100.0±0.0^{a}	100.0±0.0^{a}	100.0±0.0^{a}	100.0±0.0^{a}	100.0±0.0^{a}
	500	100.0±0.0^{a}	100.0±0.0^{a}	100.0±0.0^{a}	100.0±0.0^{a}	100.0±0.0^{a}	100.0±0.0^{a}	100.0±0.0^{a}	100.0±0.0^{a}	100.0±0.0^{a}

Results are mean ± SE values for three replicates. Means in the same column by same letter are not significantly different according to Duncan's multiple range tests ($p<0.05$).

As can be seen from presented data, PA and PK type extracts at lowest concentration did not show any relevant activity against the tested fungi, in fact PA-Ex, the most active compound, induced only 14.9% inhibition of *C. cladosporioides* IPV-F167 growth at 3 days after incubation. Crude extracts resulted completely inactive versus *C. cladosporioides* CBS 574.78 A, during the whole period of observation. At the concentration of 1000 µg mL^{-1} both extracts showed moderate inhibitory effect and *C. cucumerinum* was found to be the most sensitive species. Crude extract PA type at 2000 µg mL^{-1} exhibited potent inhibitory activity (100%) on the radial growth of *C. cladosporioides* CBS 574.78 A and *C. cucumerinum* at 3 days after incubation. At the same concentration PK-Ex induced approximately 70% inhibition of the two fungi, whereas *C. cladosporioides* IPV-F167 was found to be the least sensitive organism, in fact at 3 days after incubation PA-Ex induced 49.7% inhibition and PK-Ex 24.8%. Commercial PA, demonstrated strong efficacy on all these phytopathogenic fungi. In Figure 4.12 the sensitivity of the three strains to pure PA is graphically reported. The inhibitory activity was particularly expressed against *C. cucumerinum*, totally inhibited after 3 and 5 days of incubation, while after the 7th day, the growth was reduced by 86,1%.

Reduction of 62.4% was observed against *C. cladosporioides* CBS 574.78 A at 7th day of incubation, which results the most resistant fungus.

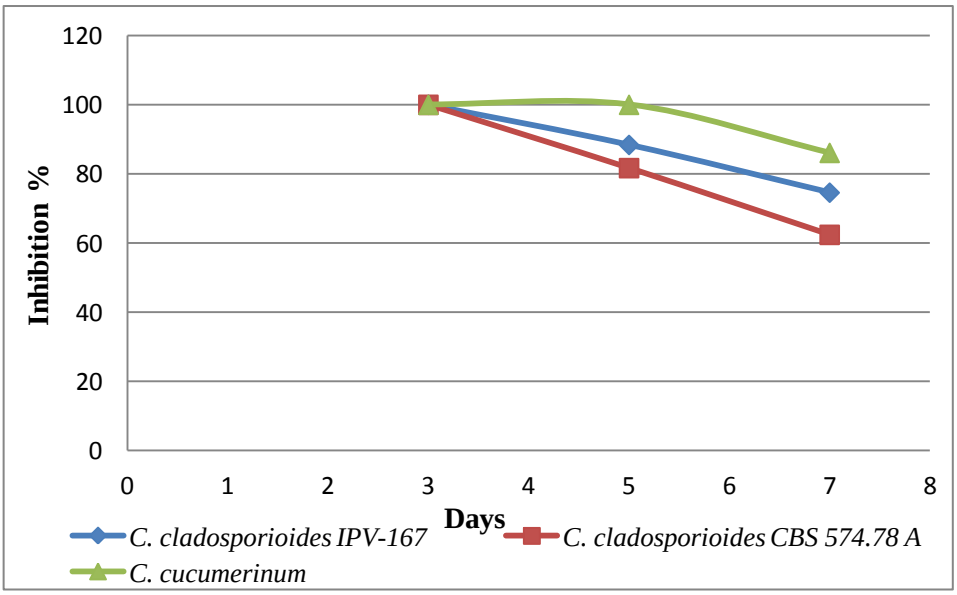


Figure 4.12 Growth inhibition induced by PA at 100 µg mL^{-1} on *Cladosporium* spp at 3, 5 and 7 days after incubation.

The inhibitory activity of PA-EO and PK-EO on mycelial growth of *Cladosporium* spp is given in Table 4.8. Both oils at lower concentration appeared to be effective against tested pathogens; however moderate inhibition values were found for *C. cladosporioides* CBS 574.78 A during the whole incubation period.

At 200 µg mL^{-1} the oils inhibited completely the growth of both strains of *C. cladosporioides* after 3 days of incubation and of *C. cucumerinum* after 5 days.

Figure 4.13 shows the dose-related activities of the oil PA-type on the three tested strains after 7 days of incubation. Inhibitory effect of PA-EO increased significantly from the concentrations of 100 µg mL^{-1} to 300 µg mL^{-1}. In the case of *C. cladosporioides* CBS 574.78, inhibition values incremented from 23.9% to 100 %, after 7 days of incubation.

At the highest concentrations employed (300, 400 and 500 µg mL^{-1}) PA-EO revealed complete inhibition on colony development for the three organisms, during the whole observation period.

At the same concentrations (7^{th} day) PK-EO inhibited totally *C. cladosporioides* CBS 574.78 A and *C. cucumerinum* while *C. cladosporioides* IPV-F167 was completely stopped at 500 µg mL, only (Figure 4.14).

It was found that essential oils of the two perilla varieties had fungistatic properties towards *C. cucumerinum* at 300 µg mL^{-1}.

PA-EO resulted also fungistatic for *C. cladosporioides* CBS 574.78 A and for *C. cladosporioides* IPV-F167 at 400 and 500 µg mL^{-1}, respectively.

PK-EO was featured by fungistatic activity towards both strains of *C. cladosporioides*, at 500 µg mL^{-1}.

The fungicidal effect was shown only versus *C. cucumerinum* by PA-EO and PK-EO at 400 and 500 µg mL^{-1}, respectively.

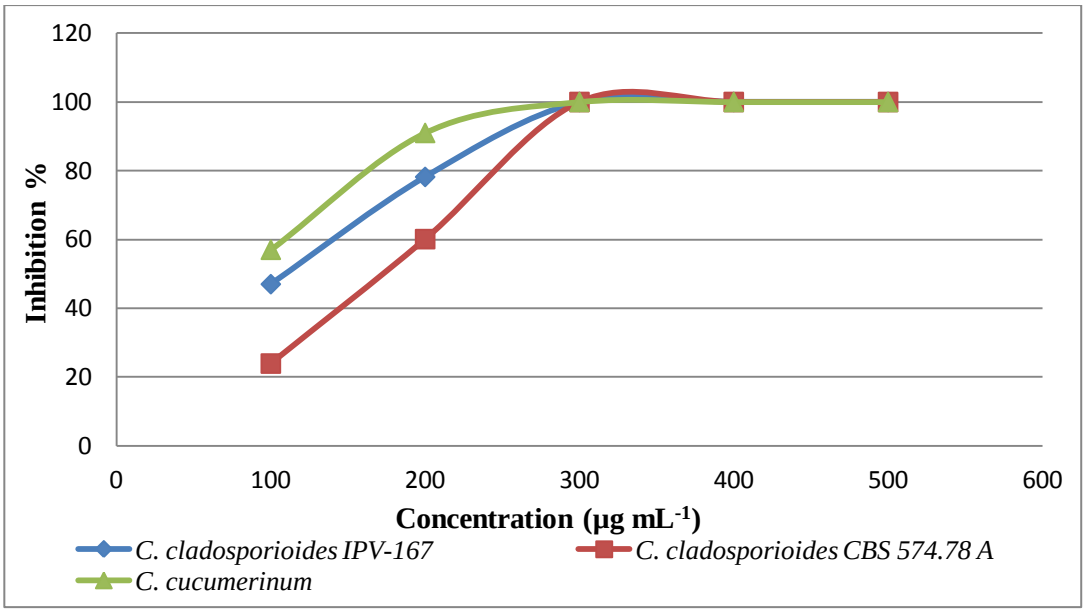


Figure 4.13 Dose-related activity of PA-EO on *Cladosporium* spp (7 days, 24°C).

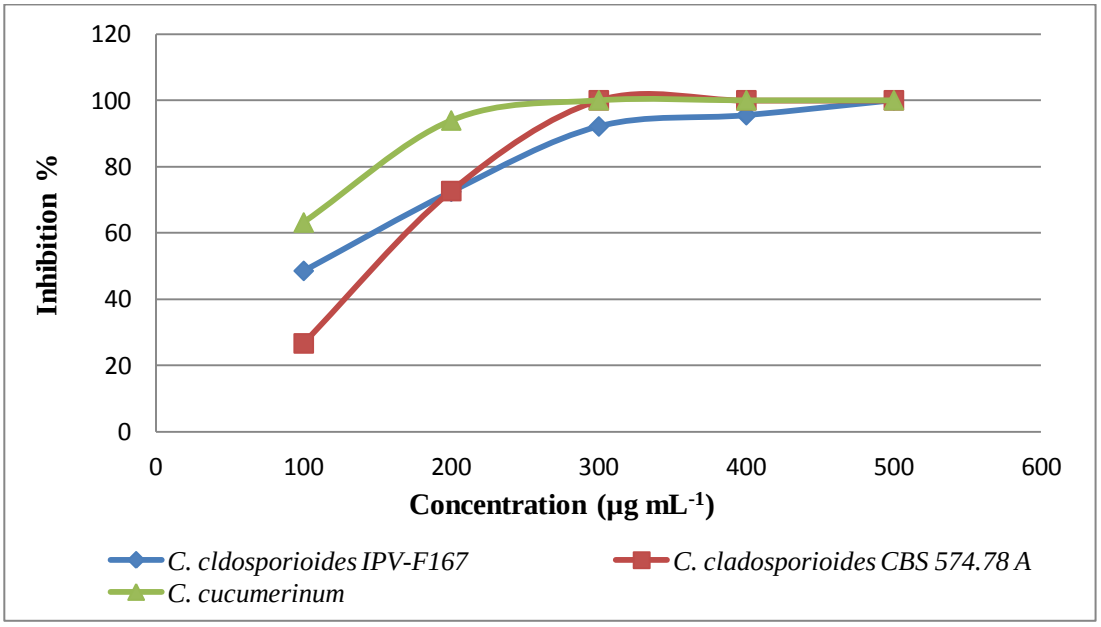


Figure 4.14 Dose-related activity of PK-EO on *Cladosporium* spp (7 days, 24°C).

4.4 *In vitro* nematicidal activity of Perillaketone

The nematodes, soil-borne plant parasites, can seriously damage the roots and therefore plants develop a deficient assimilation of water and nutrients from the soil resulting in stunted growth with reduction of crop production and severe economic losses.

In this study, the *in vitro* nematicidal activity of perillaketone (extracted from freeze-dried leaves of Korean perilla) against 2nd instar larvae juveniles of cyst nematode *H. daverti* was evaluated. The results of the trial have been reported in Figure 4.15 and Table 4.9.

At the1st observation the nematicide ethoprophos caused 100% unmotility against juveniles, PK showed a high stop of the motility (76%) and A+W a low unmotility (26%). In the observations to follow the restarting of the motility of the individuals was negligible for the chemical standard and at 13th day all the juveniles were dead. For the PK, the restarting of the motility was higher than for ethoprophos (24% at 2 th day) and at 5th day there were the first dead (22%) which gradually increased to reach the 90% at 26th day. For the A+W the first dead individuals (3%) occurred at 6th day and only at the end of the test they were the 67%. In addition, for the W the first dead juveniles (2%) were observed at 12th day to have the 20% mortality at 26th day.

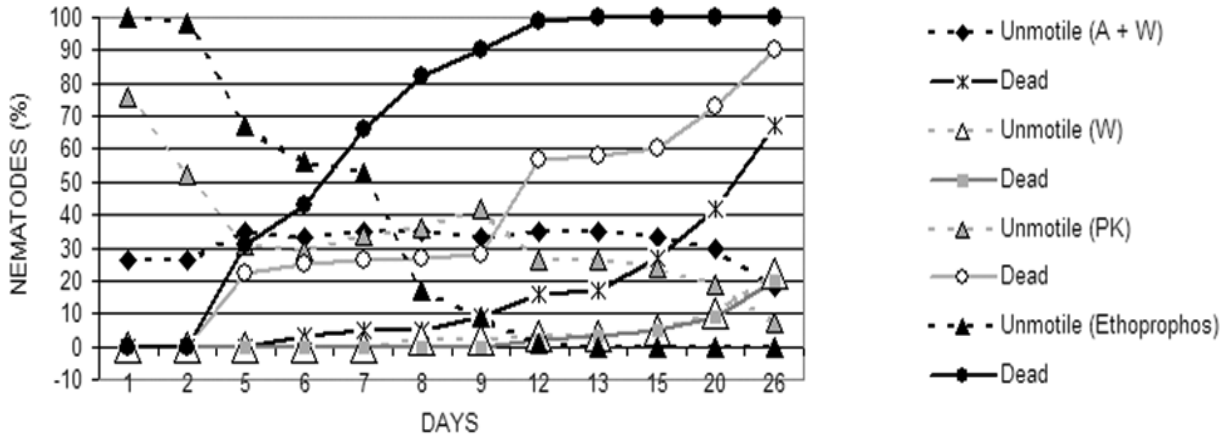


Figure 4.15 Percentage trend of mortality and unmotility of 2nd instar larvae juveniles of *Heterodera daverti*. (A+W): positive control with distilled water plus alcohol; (W): control with distilled water; (PK): perillaketone, ethoprophos.

Table 4.9 Effect of PK, at several days from contact test, on 2nd larvae juveniles of *Heterodera daverti* comparated to a positive control with distilled water plus alcohol (W+A), to a control with distilled water (W) and to ethoprophos.

Days from contact test (n.)	PK			A+W			W			Ethoprophos		
	Motile	Unmot.	Dead	Motile	Unmot.	Dead	Motile	Unmot.	Dead	Motile	Unmot.	Dead
1*	24	76	0	74	26	0	0	0	0	0	100	0
2	48 (24)	52	0	74	26	0	0	0	0	2	98	0
5	47	31 (1)	22 (22)	65	35	0	0	0	0	2	67	31
6	46	29 (1)	25 (3)	65	33	3	0	0	0	1	56 (1)	43 (12)
7	40	34 (6)	26 (1)	60	35	5	0	0	0	1	33	66 (23)
8	37	36 (3)	27 (1)	60	35	5	98	2	0	1	17	82 (16)
9	30	42 (7)	28 (1)	58	33	9	98	2	0	1	9	90 (8)
12	17	26 (13)	57 (29)	49	35	16	95	3 (3)	2(2)	0	1	99 (9)
13	16	26 (1)	58 (1)	48	35	17	94	3 (1)	3 (1)	0	0	100
15	16	24	60 (2)	40	33	27	90	5 (4)	5 (2)	0	0	100
20	8	19 (8)	73 (13)	18	30	42	81	10 (9)	9 (4)	0	0	100
26	3	7 (5)	90 (17)	10	18	67	58	22 (23)	20 (11)	0	0	100

In brackets is reported the number of the specimens passing from a state to the other.
* 1st examination, after 1 day, was directly carried out in the 4 tested samples.

4.5 Conclusion

With the growing demand for environmentally sound strategies in the control of plant pathogenic organisms and pests, the development of alternative pesticides with minimal ecological hazards has now become an imperative need. This demand is also supported by the increasing concerns over the level of pesticide residues in food and over the level of resistance of pathogens to pesticides resulting from the use of chemical pesticides (Damalas, 2011). Particularly desiderable is the discovery of novel pesticidal agents from new chemical classes, able to operate using different modes of action and, consequently, against plant pathogens with resistance to currently used agrochemicals. In this regard, natural products and plant extracts constitute an interesting resource for ecofriendly management of plant pests.

Plants produce a large and diverse array of organic compounds that appear to have no direct functions in growth and development i.e. they have no generally recognised roles in the process of photosynthesis, respiration, solute transport, translocation, nutrient assimilation and differentiation (Mazid *et al*., 2011). These secondary metabolites, once considered unimportant products, are now thought to have an important part in the plants defense system against pests and diseases, including root parasitic nematodes. Many phytochemicals have long been known to posses a broad spectrum of activity against several plant pathogenic ororganisms. For instance, cinnamon, clove, oregano and thyme essential oils showed antibacterial activity towards several phytopathogenic Gram-negative and Gram-positive bacteria and fungi. Extracts from garlic and mustard also demonstrated a wide range of antimicrobial and antielmintic property.

Number of toxic chemicals produced by plants elicits pungent sensation in mammals mediated by transient receptor potential (TRP) channels. Sensing reactive compounds are important for an organism to avoid potentially harmful environments. TRP receptors form a recently identified superfamily of cation channels that display great diversity of activation mechanisms and selectivities. Found in organisms ranging from yeast to human, transient receptor potential (TRP) channels typically contain six transmembrane domains that form a central pore, as well as differing amino and carboxyl domains that impart differential sensitivity to various sensory stimuli, including temperature, touch, pain, osmolarity, taste, pH, pheromones, and other environmental signals. Recent studies have shown that many plant chemicals interact with specific ion channels. Capsaicin (the pungent ingredient in chili peppers) produces the psychophysical sensation of “hot” or

"burning" by acting on TRPV1, whereas menthol activates TRPM8 to produce a "cooling" sensation. TRPA1 is the target of the irritant compounds in food. These include ingredients present in wasabi, horseradish, and mustard oils (isothiocyanates); garlic (allicin); cinnamon oil (cinnamaldehyde). Carvacrol, the major ingredient of oregano, and eugenol important constituent of clove oil, are both active on TRPA1.

The *in vitro* activation of TRPA1 by two secondary metabolites contained in *Perilla frutescens*, perillaldehyde (PA) and perillaketone (PK), was an interesting finding that could have potential agronomical applications.

Perilla frutescens is an annual herb commonly used in Asia as food plant, particularly appreciated for its pleasant pungent and tingling sensations. Since ancient times, perilla has been also known as an herbal medicine and used in Chinese medicine to treat a variety of diseases. Considerable attention has been given to the anti-inflammatory, anti-allergic and anti-tumor promoting substances contained in the plant.

In this study, the antimicrobial activity of crude extracts and essential oils from leaves of two *P. frutescens* varieties grown experimentally in Northern Italy, was determined against a set of common phytopathogenic microorganisms, causing widespread diseases on a variety of crops. Commercial perillaldehyde and synthetic perillaketone were also assayed *in vitro* and *in vivo* (PA, only). Pure PK was obtained both by chemical synthesis and by perilla leaves extraction. In addition, the nematicidal efficacy of pure perillaketone was evaluated against 2nd instar larvae juveniles of cyst nematode *Heterodera daverti*.

Chemical analysis by TLC, HPLC and NMR of the samples allowed the identification of PA and PK as the main secondary metabolites in the two investigated cultivars: the crisp green-leaved perilla and the smooth green-leaved Korean variety respectively, and the consequent classification in PA and PK chemotypes. The organic extracts PA and PK-type (PA-Ex and PK-Ex) and the essential oils PA and PK type (PA-EO and PK-EO) exhibited a broad spectrum of activity against tested plant patogenic organisms.

The antibacterial activity of the tested substances resulted generally scanty. Small inhibition zone was observed for PK-Ex at 2000 µg mL^{-1} against *Xanthomonas vesicatoria* 2615 and *Xanthomonas anoxopodis* pv *vignicola* 2625, only.

In vitro antifungal activity varied according to compound and target species. The sensitivity of most tested fungi to perilla compounds was evaluated as growth inhibition using the poisoned food technique. A substance may inhibit the growth of fungi either

temporarily (fungistatic) or permanently (fungicidal).

It was found that both the extracts at 2000 µg mL^{-1} exhibited fungistatic activity on *C. beticola*, moreover PK-Ex was also fungistatic against *Pyrenophora graminea* and *Pyricularia oryzae* BA43.

Results showed that among the tested samples, the essential oils appeared to be significantly more active compared with the crude extracts. It was found that for *Cladosporium cucumerinum*, both essential oils demonstrated a fungistatic activity at 300 µg mL^{-1} and a fungicidal one at 400 (PA-EO) and 500 µg mL^{-1} (PK-EO). PA-EO at the concentration of 500 µg mL^{-1} showed fungicidal activity against several pathogenic fungi such as: *Alternaria alternata* RF8, *Bipolaris oryzae*, *Cercospora beticola*, *P. graminea*, *P. oryzae* BA43 and *Pyrenophora teres*. PA-EO resulted also fungistatic for *Cladosporium cladosporioides* CBS 574.78 A and *C. cladosporioides* IPV-F167 at 400 and 500 µg mL^{-1}, respectively. On the contrary, PK-EO at the highest concentration, exhibited fungistatic activity against: *A. alternata*, *C. beticola*, *C. cladosporioides* CBS 574.78 A, *C. cladosporioides* IPV-F167, *P. graminea*, *P. oryzae*, *P. teres* and *Sclerotinia sclerotiorum*.

Essential oils efficacy could be attributed to important characteristics of their components: lipophilic and hydrophobic tendencies (Burt, 2004). Generally, these properties enables them to partition in the lipid of the fungal cell membrane and mitochondria, disturbing their structure and rendering them more permeable. Leakage of ions and other cell contents can then occur. Secondly some of the oils may affect the metabolic pathways of the microorganisms (Kumar *et al.*, 2008).

Moreover PA-EO resulted more effective than PK-EO and commercial PA revealed a high antifungal activity on mycelia growth of many tested fungi.

Among the analyzed fungi particular attention was given to *Cladosporium cladosporioides* IPV-F167. The *in vitro* antifungal activity of perilla compounds against this fungus was assessed by different methods. Semi-quantitative responses obtained by direct bioautographic TLC method, were not directly comparable with those of poisoned food technique, that was found to be more effective. In the spore germination assays both essential oils resulted active in a dose response manner, PA-EO exhibited the highest inhibitory effect. Suppression on spore germination by oil treatment could make a significant contribution to limiting the growth of the pathogen.

The *in vivo* studies showed that commercial PA had the most significant preventive

activity reducing powdery mildew disease on cucumber plants. This evidence suggests that the antifungal activity of tested essential oils is probably due to the presence of their major bioactive compounds.

Besides, *P. frutescens* demonstrated to possess good *in vitro* nematicidal activity due to perillaketone against 2nd instar larvae juveniles of cyst nematode *H. daverti*, whose mortality was 90% at the end of the test, emphasizing a significant shortening of their life.

In conclusion, this research work reported that the edible plant *Perilla frutescens*, easily cultivable, constitute a rich source of TRP bioactive compounds useful as potential alternative to agrochemicals against many phytopathogenic organisms like fungi and nematodes.

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